Macropinocytosis as Infectious Uptake Pathway of Human Species B Adenoviruses

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Summary

Cells use endocytosis to take up fluid and nutrients, translate extracellular signals into appropriate responses and maintain homeostasis. They have evolved a number of endocytic pathways to constantly communicate with their environment, retrieve extracellular information and adjust their responses to the changing environments. The cellular gateway to the outside world have been hijacked by a large variety of human and animal pathogens, including eukaryotic parasites, fungi, bacteria and viruses.

In this work I characterized the uptake mechanism of human adenovirus serotypes of the species B into epithelial and hematopoietic cells. These cell types are important targets of natural human adenovirus infections and contribute to the airway and urinary tract pathology of the species B adenoviruses.

In a first part, I focused on the entry of Adenovirus type 3 (Ad3), a species B1 human adenovirus. Ad3 is associated with epidemic conjunctivitis, and occasionally fatal respiratory and systemic disease. Ad3 binds to the membrane cofactor CD46, a member of the complement lysis cascade and alphav-integrins (a secondary receptor) which gates infectious entry into cultured cells. I show that Ad3 uses dynamin-independent endocytosis for rapid infectious entry into epithelial and hematopoietic cells. Ad3 entry spatially and temporally coincides with fluid phase uptake, and is sensitive to macropinocytosis inhibitors targeting F-actin, cholesterol, protein kinase C, the sodium-proton exchanger, and dominant negative Rac1 but not Cdc42. Infectious Ad3 macropinocytosis requires viral activation of p21-activated kinase 1 (PAK1), and the C-terminal adenoviral E1A binding protein-1-L/S (CtBP1-L/S, long and short version, respectively). CtBP1 is a transcriptional corepressor involved in membrane traffic, and coordination of innate immune responses. CtBP1 is recruited to the plasma membrane and to Ad3 containing macropinosomes by PAK1 phosphorylation coincident with transcriptional derepression. The data show that Ad3 subverts an innate endocytic immune response designed for antigen presentation, and transcriptional activation of host anti-pathogen genes. The data also suggest that the entry route used by

Ad3 appears to be independent of classical early endosomal endocytic markers like Rab5 or EEA1, and is independent of caveolar endocytosis.
In a second part I investigated the entry of Ad35, a member of the species B2 human adenoviruses. Ad35 infects urinary tracts and the kidneys, as well as the lungs in immunocompromised individuals. As Ad3, Ad35 binds CD46 for primary attachment. Like Ad3, Ad35 requires alphav-integrins for infection and activates PAK1 to involve CtBP1-L/S in the infection pathway. Similar to Ad3, dynamin played a minor role in Ad35 infection.

This work shows an involvement of a novel endocytic route in virus infection, macropinocytosis. The involvment of PAK1 and CtBP1 in species B adenovirus infections includes that the virus prepares the cellular transcription as early as the time point of cell attachment and start of signalling.

Zusammenfassung

Zellen benützen Endozytose, um Flüssigkeiten und Nahrung aufzunehmen, um extrazelluläre Signale in entsprechende Antworten umzuwandeln, und um die Homöostase zu gewährleisten. Sie haben mehrere endozytotische Wege entwickelt, um fortwährend mit der Umgebung zu kommunizieren, um extrazelluläre Informationen zu erhalten und ihre Antworten der sich ständig ändernden Umgebung anzupassen. Diese zellulären Eingänge werden von einer großen Vielfalt von Pathogenen wie eukaryotischen Parasiten, Pilzen, Bakterien und Viren benutzt.

In der vorliegenden Arbeit habe ich den Aufnahmemechanismus von menschlichen Adenoviren der Untergruppe B1 und B2 in Epithel- und Blutzellen charakterisiert. Diese Zellen sind wichtige natürliche Wirtszellen von menschlichen Adenovirusinfektionen und repräsentative Modelle für die Luftwegs- und Harnwegspathologie der Untergruppe B Adenoviren.

In einem ersten Teil konzentrierte ich mich auf den Eintritt von Adenovirus Typ 3 (Ad3), ein Adenovirus der Untergruppe B1. Ad3 ist verbunden mit epidemischer Konjunktivitis und teilweise mit systematischen Krankheiten, die selten auch tödlich enden können. Ad3 bindet an das Membrankofaktor Protein CD46, das im Komplementsystem involviert ist und an alphav-Integrine (als Korezeptor), welche den infektiösen Eintritt in die Wirtszelle unterstützen. Ich zeige, dass Ad3 Dynamin-unabhängige Endozytose für die schnelle infektiöse Aufnahme in Epithel- sowie Blutzellen benutzt. Der Eintritt von Ad3 geht einher mit der gleichzeitigen vermehrten Aufnahme von Flüssigphase und wird reduziert durch Inhibitoren der Makropinozytose, die F-Aktin, Cholesterol, Protein Kinase C, den Natrium-Protonenaustauscher inhibieren. Dominant-negatives Rac1 blockiert die Virusinfektion, nicht jedoch dominant-negatives Cdc42. Die infektiöse Makropinozytose von Ad3 benötigt die virale Aktivierung von p21-aktivierter Kinase (PAK1) und dem „C-terminal adenoviral E1A binding protein-1-L/S“ (kurz CtBP1-L/S). CtBP1 ist ein transkriptioneller Korepressor, der involviert ist im Membrantransport und in der Koordination der angeborenen Immunität. Durch PAK1-Phosphorylierung von CtBP1 wird es zur Plasmamembran rekrutiert und lokalisiert auf

Makropinosomen, die Ad3 enthalten. Mit diesem Prozeß geht wahrscheinlich die transkriptionelle Deregulierung einher. Die Daten zeigen, daß Ad3 eine angeborene Immunantwort, die für die Antigenpräsentation verwendet wird, ausnützt, um in die Zelle zu gelangen und gleichzeitig die Transkription von antiviralen Antworten der Wirtszelle reguliert. Der Eintrittsweg von Ad3 scheint unabhängig zu sein von klassischen frühendosomalen Makern wie Rab5 oder EEA1 und unabhängig von der Caveolaeendozytose.
In einem zweiten Teil untersuche ich den Eintritt von Ad35 in Wirtszellen. Ad35 stellt ein Mitglied der Untergruppe B2 der menschlichen Adenoviren dar und infiziert Harnwege, Nieren sowie die Lungen in immundefizienten Menschen. Wie Ad3 bindet Ad35 an CD46 und benötigt alphav-Integrine für die Infektion. Auch Ad35 aktiviert PAK1 und benötigt CtBP1-L/S für die Infektion. Auch beim Eintritt von Ad35 spielt Dynamin eine untergeordnete Rolle.

Diese Arbeit zeigt, daß Makropinozytose als infektiöser endozytotischer Weg von Viren verwendet wird. Bei diesem Prozeß sind PAK1 und CtBP1 involviert und wir vermuten, daß der Virus bereits beim Andocken an die Wirtszelle und Beginn des Signalling die zelluläre Transkription verändert.

Introduction

Endocytic pathways

Endocytosis is the cellular uptake mechanism of proteins and lipids, fluid phase and extracellular ligands from the cell surface. It is involved in many different processes, e.g. nutrient uptake, synaptic vesicle recycling and establishment of cell polarity. The endocytosed cargo is transported in membrane-surrounded carriers targeted to specific destinations within the cell.

So far, many different pathways have been reported, some are only present in specialized cells and limited to certain cargoes (a cargo can be as different as a lipid, a receptor-ligand complex or a whole organelle like an endosome), some operate constitutively in all cell types, and others are only induced upon requirement. The most important pathways are discussed below, they are reviewed in (Conner and Schmid 2003, Helenius and Marsh 2006, Mayor and Pagano 2007). They can be operationally grouped into clathrin-dependent and clathrin-independent uptake routes based on the notion that the clathrin pathway is the best characterized endocytic uptake route. A model of the most important, currently known endocytic pathways is depicted in figure1.

Viruses are obligatory intracellular parasites. They have to overcome the barrier of the plasma membrane to reach the cytosol. Many viruses have evolved to use endocytosis as a shuttle pathway into cells. This has allowed studies of basic cellular mechanisms with the help of viruses. They are attractive tools to study endocytosis, due to their simple composition and architecture. Infection serving as a functional endpoint for cell biologists and virologists can readily be monitored using reporter genes or antibodies against virally encoded proteins, or viruses can be labeled with fluorophores and tracked both extracellularly and intracellularly. The precise nature and subcellular localization of viruses can be determined in high resolution electron micrographs.

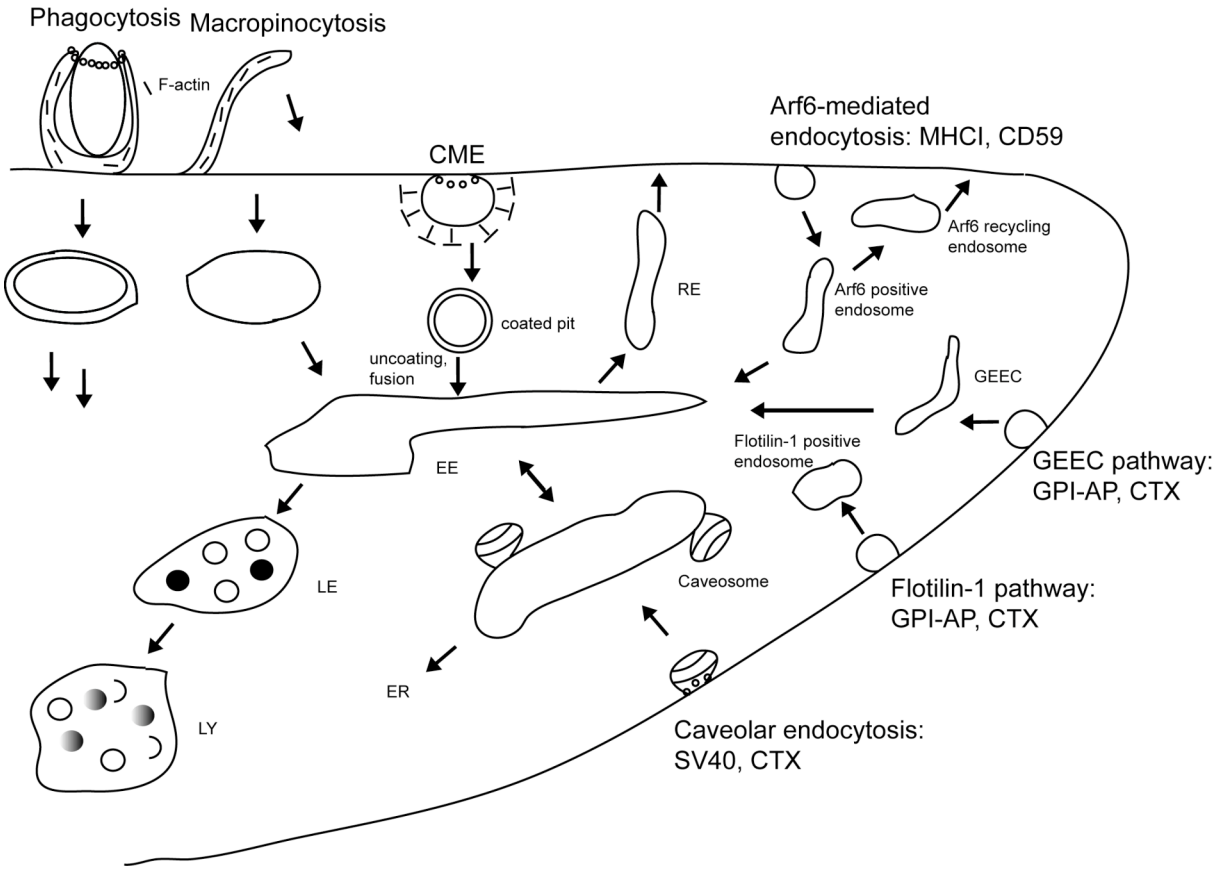

Figure1: Different endocytic pathways. CME = Clathrin-mediated endocytosis; GEEC = GPI-AP enriched early endosomal compartment; EE = early endosome, LE = late endosome; LY = lysosome; RE = recycling endosome; CTX = cholera toxin B

Clathrin-dependent endocytosis:

Clathrin-mediated endocytosis (CME) is the best characterized endocytic pathway. It is involved in the internalization and recycling of receptors participating in signal transduction events and in nutrient uptake (for reviews: Johannes and Lamaze, 2002; Conner and Schmid, 2003). Internalization can occur either constitutively or in response to certain stimuli, e.g. epidermal growth factor (EGF) binding to the EGF receptor.

CME concentrates transmembrane receptors and their bound ligands into so-called coated pits that are formed by a stepwise assembly of cytosolic coat proteins (Robinson 1987). The most important component of these structures is clathrin, a protein that assembles into trimers of three heavy and light chains to form a three-legged assembly called triskelion. At the beginning of CME, adaptor protein 2 (AP-2) is targeted to the plasma membrane binding the lipid Phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$) where it mediates clathrin assembly into curved lattices

(Brodsky et al., 2001; Kirchhausen et al., 1999 and Robinson 1987). Other proteins are required for the assembly, like amphiphysin, or Eps15. The clathrin assembly lymphoid myeloid leukemia (CALM) protein is another clathrin associated protein that plays an is important role in regulation of clathrin internalization machinery (Dreyling et al., 1996; Tebar et al., 1999).

The multidomain GTPase dynamin is recruited to the neck of coated pits where it mediates membrane fission and the release of the clathrin coated vesicles (CCVs). Dynamin is also thought to act as an organizer much like the SAR1 GTPase in CopII mediated sorting in secretory pathway (Schmid et al., 1998 ; Jones et al., 1998). It is possible to inhibit CME in cultured cells at the stage of vesicle budding by overexpression of dominant-negative K44A mutant defective at GTP hydrolysis (Damke et al., 1994). Similarly, the drosophila shibire mutant encoding a temperature sensitive dynamin function, is impaired in neutrotransmitter release in neuronal synapses due to defective vesicular budding (Poodry and Edgar 1979; Koenig et al., 1989). In contrast, the genetic knock out of the neuronal isoform of dynamin in mice gives raise to apparently normal mice (Ferguson et al., 2007), suggesting that blocking dynamin function by nonfunctional proteins is far more effective and less prone to back up mechanisms replacing dynamin.

AP-2 is not necessarily needed in CME as recently shown for the uptake of EGF (Motley et al., 2003). In this study transferrin uptake was decreased in cells which were AP2 depleted by siRNA, whereas EGF uptake was not reduced. Transferrin and EGF are commonly used as typical cargoes internalized by CME (Anderson et al., 1982; Hopkins and Trowbridge 1983; Carpenter and Cohen, 1979). It was suggested that other adaptor proteins than AP-2 are involved in CME.

Many different viruses use clathrin coated pits to enter mammalian cells. As an example for enveloped viruses, Semliki-Forrest virus, Influenza virus, vesicular stomatitis virus, and as a non-enveloped example, Adenovirus type 2, have been shown to enter cells by clathrin-mediated endocytosis (Helenius et al., 1980; Matlin et al., 1981; Matlin et al., 1982; Chardonnet and Dales, 1970 and Meier et al., 2002). More detailes about clathrin-mediated endocytosis of Adenovirus will be disscussed under “Adenovirus endocytosis”.

Early endosome

CCVs loose their coat soon after internalization. They fuse with so-called early endosomes (EE) in a Rab5-dependent manner and deliver their cargo (Gorvel et al., 1991). Intracellular fusion events require the common components N-ethyl maleimide-sensitive factor (NSF) and alpha-soluble NSF attachment protein (alpha-SNAP). These factors interact with a membrane receptor complex called “SNAP receptors” or shortly “SNAREs”. SNAREs are present on the vesicle (termed v-SNARE) and at the target membrane (t-SNARE). Vesicle fusion is mediated by coiled-coil protein that initalize the attachment of the membranes destined to fuse. Small GTPases of the Rab family (e.g. Rab5 for the fusion event between vesicles of the clathrin-mediated pathway and the early endosome and homotypic early endoeome- endosome fusion) that cycle between activated (GTP-bound) and inactivated form (GDP-bound) and SNAREs determine the specificity of the vesicle fusion event. GTP-hydrolysis of the Rabs mediates the fusion. (Clague et al., 1999 and Rothman, 1994; Zerial and Stenmark, 1993).

The compartment reached by the cargo within the first 5 minutes after internalization is termed EE (Marsh et al., 1983, Schmid et al., 1988). Typical markers of EE are Rab5, Early Endosome Antigen 1 (EEA1) and the cargo transferrin together with its receptor, transferrin receptor (Chavrier et al., 1990; Simonsen 1998; Zerial 2001). From EE cargoes can be further transported to either recycling compartments or degradative pathway involving late endosomes (LE) and lysosomes.

The lumen of the EE is mildly acidic (pH between 6.0 and 6.5), caused by the activity of the vacuolar ATPase. Due to the lower pH dissociation of some receptor-ligand complexes occurs. While the receptors are sorted in tubulo-vesicular structures away from the bulk of the EE contents to recycling endosomes, the soluble cargoes gradually reach more acidic compartments with an increasingly degradative environment. For example, low density lipoprotein (LDL) binds to its receptor at neutral pH at the plasma membrane and dissociates in early endosomes. The receptor is recylcled by either a direct Rab4 dependent fashion or indirect and slower through Rab11 and Rab4 positive endosomes (Gruenberg, 2001; Maxfield and McGraw, 2004).

As a different example, EGF bound to the receptor is further sorted out into vesicles invaginating from the endosomal membrane into the lumen of the early endosomal vacuole, followed by the subsequent lysosomal degradation. An important sorting signal is monoubiquitinylation (Hicke and Dunn, 2003; Katzmann et al., 2002). Clathrin coats on the endosomal membrane provide a scaffolding platform to collect ubiquitin binding proteins like e.g. Hrs (Raiborg et al., 2006; Sachse et al., 2002). Hrs binds ubiquitinylated cargo and recruits the multisubunit ESCRT complex consisiting of ESCRT-I, -II, and –III that function downstream of Hrs in internal vesicle formation.

Late endosomes and lysosomes

After passing through early endosomes cargoes destined to the degradative pathway are transported to late endosomes (LE). LE have a lower pH (below 6) and typical markers are Rab7, Lamp1, Lamp2 and the lipid lysobiphosphatidic acid (LBPA, Kobayashi et al., 1998). The first LE are reached by endocytic tracers within 10 to 15 min, however, most of the typical markers reach the LEs after 30 min. LE contain several internal vesicles and are therefore referred as multi-vesicular bodies (MVBs). In LEs degradation starts. Lysosomes overlap with LEs to some extent and are the final site of degradation of internalized molecules (Kornfeld and Mellman, 1989). Morphologically the lumen of lysosomes is electron dense with internal vesicles at different degradation stages. The pH is lower then in LE, about 5.5 (Sachse et al., 2002).

How is cargo transported between early and late endosomes? Two model have been suggested to answer this question, the maturation and the vesicle shuttle model (Helenius et al., 1983).
The maturation model assumes a *de novo* formation of early endosomes at the cell periphery by fusion of incoming primary endocytic vesicles to progress later on to become late endosomes. First the early endosome functions as a sorting station for incoming cargo, in either routing it to the recycling compartment or retaining the cargo that is destined for degradation. Later on, possibly by increasing the intraendosomal pH, it moves towards the perinuclear region where it fuses with vesicles from the Golgi complex carrying lysosomal hydrolases and proteases to become a late endosome.

The alternative is the vesicle shuttling model. Here, both early and late endosomes are different organelles, with defined locations and functions. Cargo is moving between them in vesicles pinching off from the early endosome and fusing with the late endosome. (Gruenberg and Stenmark, 2004).
A study by Rink (Rink et al., 2005) favoured the maturation model. Using fast live-cell imaging and image-analysis algorithms they found that Rab5 fluctuated dynamically on individual endosomes. Degradative cargo (LDL in this study) was concentrated in larger endosomes migrating towards the cell centre and Rab5 was lost and at the same time replaced by Rab7.
However, in a study from Vonderheit and Helenius it was suggested that early endosomes are stable compartments sorting cargo in organized sites of the early endosome to be transported to late endosomes. Rab7 is recruited to this site and Rab7 positive vesicles are formed that fuse with late endosomes (Vonderheit and Helenius, 2005).

Clathrin-independent endocytosis

Apart from the classical CME several clathrin-independent uptake mechanisms are emerging. Different cargoes are internalized by these pathways and are sometimes hijacked by bacteria and viruses to gain access to the cells. Some of them are dependent on lipid-rafts, e.g. plasma membrane microdomains rich in cholesterol and sphingolipids.

Caveolar endocytosis

Caveolae are membrane invaginations smaller than clathrin-coated pits (50-80 nm in diameter) and normally do not pinch off. They are coated with caveolin-1 and seen in many, but not all cell types. They are absent e.g. in certain T-cells. One of the best studied ligand of caveolar endocytosis is cholera toxin B (CTB). CTB is internalized within caveolae whereas at high concentration CTB can take other endocytic pathways as well (Nichols, 2002.). It has been shown that in certain cell types the infectious entry pathway of simian virus 40 (SV40) involves caveolae-mediated uptake which is gated by tyrosine phosphorylation at the plasma membrane (Anderson et al., 1996; Pelkmans et al., 2001; Pelkmans et al., 2002). Furthermore, cargo internalized by this pathway is transported to caveosomes, a preexisting

compartment with neutral pH (Pelkmans et al., 2001). Caveosomes are accessed also by non-caveolar uptake routes. Further transport from the caveosome is either directed to the endoplasmic reticulum (Pelkmans et al., 2001) or to early endosomes depending on the activation signal (Pelkmans et al., 2004). This was first shown by SV40 (Kartenbeck et al., 1989). An interesting study in cells lacking caveolin-1 from caveolin-1 knockout mice (Damm et al., 2005) revealed a more rapid internalization of SV40 by a pathway independent of clathrin, caveolin and dynamin. In tight fitting invaginations the virus is internalized to a caveosome-like compartment. It is thought that both the caveolar and the caveolar-independent uptake pathways of SV40 (co-) exist in normal cells.

Macropinocytosis

Macropinocytosis is a receptor and coat-independent form of endocytosis. It is stimulated by growth factors or pathogens like Salmonella or Adenovirus type 2. Its underlying signalling cascade includes the sequential activation of PLCgamma and PKC, further stimulation of Phosphatidylinositide-3-kinases (PI3Ks) and Rho-family GTPases (Rac and Cdc42) which trigger the actin-driven formation of membrane ruffles through binding and activation of the p21-activated kinase (PAK) which in turn activates other down stream targets, and together with activated RhoGTPases leads to ruffling at the cell surface. Upon closure of the so called lamellipode macropinosomes are formed. They comprise large volumes of extracellular fluid. These large dynamic structures are of a size of several µm and move towards the nucleus or can be recycled back to the plasma membrane (Conner and Schmid 2003; Swanson and Watts, 1995). Interaction with other endocytic vesicles are used for exchange of internalized fluid, cargo and membrane lipids, such as late endosomes e.g. in antigen-presenting cells. However, alternative pathways are possible, Meier and Greber, 2003 suggested to differentiate two different kinds of mechanisms, recycling and processive macropinocytosis (Meier and Greber, 2003).

In the recycling type of macropinocytosis stimulated by growth factors like EGF or PDGF (platelet-derived growth factor) the internalized fluid and surrounding membrane is recycled back to the plasma membrane, in the processive macropinocytosis trafficking within the cell and fusion with further

endosomes/lysosomes occurs. This type of macropinocytosis is found in cells stimulated by certain bacteria like Salmonella. It is related to phagocytosis but differs in the aspect that in phagocytosis actin protrusions are formed around a cargo and the use of the large GTPase dynamin in the fission of the phagosomes which is not needed in macropinocytosis. Actin-polymerising and depolymerising agents such as Cytochalasin D are drugs which target macropinocysosis. In addition, protein kinase C inhibitors and the Na^+/H^+ exchanger inhibitor amiloride are typical drugs inhibiting macropinocytosis. The key-players in macropinocytosis are listed more detailed below.

Bacterial induced macropinocytosis can be divided into zipper (e.g. Listeria) and trigger (e.g. Shigella or Salmonella) type (Veiga and Cossart, 2006). Listeria is expressing proteins on the surface that interact with cellular receptors and induces signalling cascades that result in plasma membrane enclosure of the bacteria. Actin polymerization and modest membrane extensions ensure bacterial internalization. Shigella on the other hand do not bind to cellular receptors but inject protein effectors that interact with the actin cytoskeleton using the type III secretion system. This system is a complex protein machinery that ensures direct secretion from the bacterial cytoplasm into the cytosol of the mammalian cell.

Actin polymerization

Actin is the monomeric subunit of microfilaments and its polymerization and reorganization is involved in many different cellular processes e.g. cell migration, cell division, but also resphaping of the plasma membrane, e.g. in different endocytosis mechanisms and later on in vesicle and organelle transport. In mammalian cells, caveolar-uptake, macropinocytosis and phagocytosis need actin assembly/dissasembly for internalization (Kaksonen et al., 2006). With particular cargoes, CME is thought to involve actin assembly during the time of plasma membrane invagination and the scission step, thus actin polymerization may function in the invagination, constriction and scission function (Kaksonen et al., 2006). In macropinocytosis and phagocytosis actin polymerization is needed for lamellipode formation. Actin filaments are the main structural component of this protrusion (Small et al., 2002).

Na^+/H^+-exchanger

The family of Na^+/H^+-exchanger (NHE) consists of 6 known isoforms (NHE1 to 6). They mediate the electroneutral countertransport of H^+ for Na^+ across cellular membranes and are therefore responsible for cellular pH homeostasis and regulate organellar pH. It was shown that amiloride, an inhibitor of NHEs decreases the fluid phase uptake stimulated by EGF or by Adenovirus type 2 without affecting the endocytosis of EGF or transferrin (West et al., 1989 and Meier et al., 2002). It is, however, unknown how NHE is involved in macropinocytosis. An important question is, whether the NHEs are involved in signalling or whether the intracellular pH is important for mediating macropinocytosis (Orlowski and Grinstein, 1997; Szaszi et al., 2000).

PI3K

These kinases are involved in cell growth, cell migration survival and nutrient uptake (Wymann et al., 2003; Wymann and Marone, 2005; Cantley, 2002). PI3K can be activated by growth factor receptor tyrosine kinases, integrin-dependent cell adhesion and by G protein coupled receptors.

Originally PI3Ks were defined on the basis that they phosphorylate the 3-position of the inositol ring in one or more phosphoinositide substrates (e.g. PtdIns(4,5)P_2 phosphorylation leads to PtdIns(3,4,5)P_3). Signalling molecules that contain a pleckstrin homology (PH) domain accumulate at sites of PI3K activation, particularly at the plasma membrane, by binding to PIP(3,4,5)P_3. PI3K downstream targets are PH domain containing PKB (also called Akt) and in respect to macropinocytosis, protein kinase C PKC.

PKC

PKC isoforms are involved in many different processes, including cellular migration and change of cytosceleton morphology. The PKC family consists of three groups, classical (PKCα, β and γ), novel (PKCδ, ε, η and θ) and atypical (PKCλ and ξ) (Mellor and Parker, 1998). The classical PKCs are activated by phosphatidylserine (PS) in a Ca^{2+}-dependent manner. They bind diacylglycerole (DAG) that is produced by e.g. phospholipases, increasing the activity (Takai et al., 1979). Phorpholesters like PMA mimick the action of DAG and stimulate PKCs (Castagna et al., 1982). Novel PKCs

are Ca^{2+}-insensitive, but are still activated by DAG or PMA in the presence of PS. (Ono et al., 1988). The atypical PKCs are Ca^{2+}-insesitive and are not activated by PMA or DAG (Ono et al., 1989).

PKC is kept in an inactive conformation by binding of the pseudosubstrate sequence to the substrate-binding cavity. DAG and/or Ca^{2+} recruits PKC to the plasma membrane where the energy needed for the release of the pseudosubtrate is provided, allowing substrate binding and phosphorylation. However, PKC must first be phosphorylated before it can interact with downstream partners, which is done by the upstream kinase PDK-1 (Newton, 2003). Subsequent dephosphorylation inactivates PKC.

Rho-GTPases

Rho-GTPases are involved in many different stages of vesicular trafficking. Most importantly for endocytosis, they are implicated in localized actin dynamics in cells. The most important RhoGTPases in the context of endocytosis are Rac, (stimulates veil-like protrusions of the plasma membrane, so-called lamellipodia), Cdc42 (induces finger-like extensions, called fillopodia) and RhoA (stimulates the formation of actin bundles, so-called stress fibers). Overall these proteins are key regulators in actin dynamics. All GTPase cycle between an active (GTP)- and an inactive (GDP)-bound state. In the GTP-bound state Rho-GTPases bind to down-stream molecules and activate them, as an example Rac1 and Cdc42 bind to PAK1 (Manser et al., 1994). RhoGTPases can localize to the plasma membrane where they are activated locally by nucleotide exchange factors (GEFs), e.g. as an example Rac activity was monitored in live cells by a FRET approach (Kraynov et al., 2000).

PAK

p21-activated kinases (PAK1, 2 and 3) are a group of serine/threonine kinases orignially identified as targets of the RhoGTPases Rac and Cdc42 (Manser et al., 1994; Sells et al., 1997). In their GTP-bound form, Rac and Cdc42 bind to a specific p21-binding site relieving the influcence of an adjacent autoinhibitory kinase domain. Autophosphorylation occurs, PAK is auto-actived and phosphorylates downstream targets like Lim-domain-containing protein kinase (LIMK) which in turn phosphorylates and inactivates cofilin (Edwars et al., 1999; Arber et al., 1998; Yang

et al., 1998) which is involved in actin depolymerization and must be turned off for proper macropinocytic activity.
Furthermore PAK is also a signaling element in the control of cell motiliy (Sells et al., 1999; Kiosses et al., 1999).

An interesting direct link to transcription was found as PAK1 phosphorylated C-terminal binding protein (CtBP1), a transcriptional corepressor, which was in turn translocated from the nucleus to the cytoplasm (Barnes et al., 2003). Gene transcriptonal activity that was under control of CtBP1 was changed upon PAK1-phosphorylation of CtBP1.

Phagocytosis

Phagocytosis is the uptake mechanism of either large pathogens like yeast or bacteria or the removal of large cell debris such as remnants of dead cells or dust particles. It is a highly regulated process and induced through specific cell-surface receptors and signalling cascades involving Rho-family GTPases (Aderem, 1999 and Hall, 2000). It is a process very similar to macropinocytosis and existing particularly in certain cell types, like macrophages or neutrophiles. Phagocytosis is either mediated by the Fc receptor that binds the conserved Fc domain of immunoglobulins, called zippering or type I phagocytosis, or by the complement receptor proteins, called sinking or type II phagocytosis (Le Cabec et al., 2002; Meier and Greber, 2003). Phagocytosis involving Fc receptor is very similar to macropinocytosis. First, opsonized particles (particles carrying an e.g. IgG coat) bind to the Fcγ receptor and subsequent rapid internalization is mediated by sequential activation of PLCgamma and PKC, and activation of Ras and Src together with PI3K to promote F-actin dynamics by Rac1 and Cdc42. In the sinking type of phagocytosis involving complement receptor 3, a different src family tyrosine kinase, namely Hck is involved whereas it is not needed for the zippering type of phagocytosis. Another difference is that in the sinking type of phagocytosis RhoA instead of Rac1/Cdc42 is needed for the actin remodeling (Underhill and Ozinsky, 2002; Cabec et al., 2002; Niedergang and Chavrier, 2005; Hall et al., 2006). Final internalization in both types of phagocytosis needs the recruitment of dynamin for the final fission step.

GEEC-pathway

Surface proteins that carry a glycophosphatidyl-anchor (so-called GPI-APs) are targeted to lipid rafts. GPI-modified proteins have been found to be taken up together with the fluid phase tracer dextran in a Cdc42-dependent fashion ending up in a compartment called GEEC (GPI-APs-enriched early endosomal compartment, Sabharanjak et al., 2002). Later, GPI-APs have been found to partly colocalize with early endosomal marker like EEA1 showing that after passing through the GEECs cargo is further transported to early endosomes. Colocalization between GPI-APs with transferrin after passing through GEECs showed as well that cargo internalized by the clathrin-independent GEEC-pathway is merged later with the cargo taken up in a clathrin-dependent fashion. Additionally the part of cholera toxin B that is taken up independently of caveolar and CME seems to colocalize with GPI-APs. This uptake mechanism is independent of the fission activity of the large GTPase dynamin and therefore operationally defines a distinct entry port into cells (Sabharanjak et al., 2002, Mayor and Riezman, 2004). Cdc42 but not Rac, nor RhoA mediate this uptake mechanism, which is a discriminating feature of the GEEC pathway.

Arf6

The small GTPase Arf6 has been shown to regulate the lipid-raft dependent uptake of MHCI and the GPI-anchored protein CD59 to a distinct, Arf6 positive endosomal structure in HeLa cells (Radhakrishna et al., 1997). It is initially separated by the CME pathway but merges later on with some of the CME cargo in early endosomes. A portion of the cargo is further transported to Arf6-positive recycling endosomes that fuse back to the plasma membrane. Arf6 activation is not needed for cargo internalization but its subsequent inactivation is a prerequisite for fusion of the Arf6-endosomes with early endosomes (Naslavsky et al., 2003 and 2006). Overexpression of constitutively active Arf6 (Q67L) leads to membrane ruffling and macropinosome formation (Brown et al., 2001). The pathway leading to GEECs was found to function independent of Arf6, as GEECs were negative of Arf6 (Kalia et al., 2006).

Flotilin

A clathrin and dynamin independent uptake mechanism was recently found to be supported by the protein flotillin-1 and flotillin-2. GPI-anchored protein, fluid phase marker dextran and to some extent cholera toxin B were found to be colocalizing with flotilin-1 positive endosomes (Glebov et al., 2006). By interfering with siRNA against flotilin-1 both CD59 and cholera toxin B showed decreased internalization rates. Flotillin-1 and flotillin-2 coassembly induces membrane curvature and invaginations of the plasma membrane resembling caveoleae (Frick et al., 2007).

WAVE endocytosis

In difference to lamellipodia, actin-rich structure that help to pull or push a cell forward, there are circular dorsal ruffles/waves produced by e.g. PDGF-stimulation which occur transiently in a single “wave” across the cell surface. These events have been postulated as being involved in preparation of a static cell for motility and possibly function as well in macropinocytosis (Buccione et al., 2004). Circular dorsal ruffles/waves are actin-based structures that are transiently formed. They might be involved in internalization of receptor tyrosine kinases e.g. EGF after EGF-receptor binding (Orth et al., 2003). Importantly the uptake occurs independent of clathrin- or caveolar endocytosis therefore wave endocytosis might provide an novel and efficient uptake mechanism for receptor tyrosine kinases. An important signalling kinase that have been localized to circular ruffles is PAK1 (Dharmawardhane et al., 1997; Dharmawardhane et al., 2000), therefore a close relationship with macropinocytosis is proposed (Buccione et al., 2004).

As with all the lipid raft supported pathways, future studies are needed to shed more light into these uptake processes to gain more information about the detailed mechanisms.

Alternative fission machinery for Dynamin: CtBP1-L/S

The large GTPase dynamin is involved in most of the endocytic and also some secretory fission events (McNiven, 2006). Some of the above mentioned pathways, e.g. macropinocytosis and the uptake mechanism involved in the GPI-APs internalization were shown to function independent of dynamin. Therefore other fission machineries may exist. One such protein involved in dynamin independent fission is C-terminal binding protein 3/Brefeldin-A ribosylated substrate (CtBP3/BARS)(Bonazzi et al., 2005). It is a splicing form of CtBP1, lacking 11 N-terminal amino acids. The two forms are hereafter named CtBP1-L/S (long and short, respectively). CtBP1 is a transcriptional corepressor binding the C-terminal part of adenoviral protein E1A (Chinnadurai, 2002). It functions by binding to distinct histone deacetylases. However, repression at some promoters occurs independent of histone-deacetylation activity since it is not affected by the inhibitor trichostatin A, a drug inhibiting histon-deacetylases (Berk et al., 2005).

CtBP1-S is observed at Golgi membranes (Hidalgo and Carcedo et al., 2004) and is involved in mitotic Golgi partitioning and membrane trafficking (Colanzi et al., 2007). Additionally it is found in dense bodies of synaptic termini where it may have a role in membrane organization (Gallop et al., 2005). In mammalian cells, two CtBP genes exist, both having a shorter splice form (CtBP1-L/S and CtBP2-L/S, Verger et al., 2006). Only CtBP2-L contains a nuclear import signal NLS that is not present in its shorter splicing form CtBP2-S. Although CtBP1 and 2 are encoded by different genes, they have partly overlapping transcriptional functions in tumorigenesis, development, apoptosis, cell differentiation, cell cycle regulation and viral infection (Berk, 2005). CtBPs heterodimerize and shuttle between the cytoplasm and the nucleus depending on posttranslational modifications and PDZ containing proteins (Barnes et al., 2003; Verger et al., 2006). Upon phosphorylation by PAK1 CtBP1 was found to be exported from the nucleus in line with transcriptional changes of a reporter gene.

CtBPs were thought to act as dehydrogenases since they share sequence homology with dehydrogenases in a conserved Rossman fold motif with the binding side for NADH/NAD+ (Rossman 1974). However, the dehydrogenase activity was either

reported as being low, or, it was even suggested that NADH/NAD+ binding affects rather the structure per se and is not needed for the dehydrogenase activity (Zhang et al., 2002). NADH binds more effectively to CtBP than NAD+ and upon change of the nuclear NADH/NAD+-ratio structural changes in CtBPs are caused that may change the cellular distribution of the proteins. Exposing cells to compounds changing the cellular redox state revealed different binding pattern to e.g. E1A and a change of transcription could be monitored. Therefore a redox-sensing function for transcription was proposed and it is thought that CtBP evolved from dehydrogenases in a matter that resulted in the loss of the enzymatic activity but retention of the capacity to be regulated by the NADH/NAD+ ratio (Zhang et al., 2002).

Adenovirus

Adenoviruses are grouped into four genera, the Mastadenovirus, Aviadenovirus, Atadenovirus and Siadenovirus due to serological destinction. Aviadenoviruses are limited to viruses infecting birds whereas the Mastadenovirus genus includes human adenoviruses (Ads) and adenoviruses from other species like bovine, porcine, canine and many more (Benkö et al., 2005 and Fields Virology). Atadenoviruses have a broad host range including animal species (snakes, lizards, goose, duck, chicken, possum) from different vertebrate classes (reptilia, birds, mammals). In respect to other Adeonviruses they have different genomic organization and capsid protein complements. They have several unique proteins, some showing very little similarity to their suspected counterparts in other adenovirus genera. The genus of Siadenovirus consists of only two member, frog adenovirus 1 and turkey adenovirus 3 and were therefore isolated from an amphibian (frog) and birds (turkey and chicken), respectively.

So far 51 different subtypes of human adenoviruses have been characterized. They are divided into 6 species A to F according to their hemaglutination ability (Fields Virology). The species B is further subdivided into the group B1 (infecting mostly respiratory organs: Ad3, Ad7, Ad16, Ad21 and Ad50) and B2 (infecting urinary tract and the kidneys: Ad11, Ad14, Ad34 and Ad35). The adenoviruses of the species B bind to CD46 receptors and can infect blood cells (Li and Wadell, 1988; Schmitz et al., 1983; Gaggar et al., 2003; Sirena et al., 2004, Segerman et al., 2003). The two

members of the species C adenoviruses, Ad2 and Ad5, belong to the best-studied adenoviruses and are widely used vectors for gene-therapy approaches or as oncolytic agents (Kirn et al, 2002; Kochanek et al., 2001). These viruses bind the *C*oxsackie B virus and *A*denovirus *R*eceptor, shortly *CAR* (Greber and Gastaldelli, 2007).

Adenovirus pathology

Adenovirus have a broad range of human pathology: They infect either upper or lower respiratory tracts with the two members Ad3 and Ad7 causing exacerbations of asthmatic conditions, and morbidity and mortality (Gray et al., 2000; Kim et al., 2003), urinary tract (Species B2 Ads like Ad11 or Ad35) and furthermore, Ad3 and Ad37 (Species D) has been associated with epidemic conjunctivitis (Shiao et al., 1996). Member of the Species D have further been associated with strong eye infections, like e.g. keratojunctivitis (Wigard et al., 1983; de Jong et al., 1981).
The causative agent of enteric infections are the two member of the Species F Ads, Ad40 and Ad41, respectively (Uhnoo et al., 1990). They contain two different fiber, a long and a short one, and they are acid and heat stable (Kidd et al., 1981; Favier et al., 2002).

Virion structure

Ads are non-enveloped icosahedral particles measuring 70 to 100 nm in diameter (Horne et al., 1959). Virions consist of a capsid surrounding a double-stranded DNA core of a size up to 36 kb with two terminal proteins, condensing proteins V and VII and about ten copies of the cysteine protease p23. The capsids are composed of 240 subunits of hexons and 12 pentons (Ginsberg et al., 1966). Each penton contains a pentameric base to which the trimeric fibers are bound. These fibers, responsible for receptor binding, are of different length among the serotypes (Norrby, 1966), e.g. Species B Ads having shorter fiber than Species C Ads: Ad3 has a fiber with a length of 16 nm and 5 shaft repeats whereas the fiber of Ad2 is 37 nm long and contains 11 shaft repeats (Ruigrok et al., 1990). The penton base carries an arginine-glycine-aspartate (RGD) motive that is exposed and responsible for coreceptor integrin binding (Stewart et al., 1997; Wickham 1993; Cuzange et al., 1994). Viron models of

prototypic species B and C Ads are depicted in figure 3, however, the kink in the fiber protein of species C Ads is not shown (Wu et al., 2004).

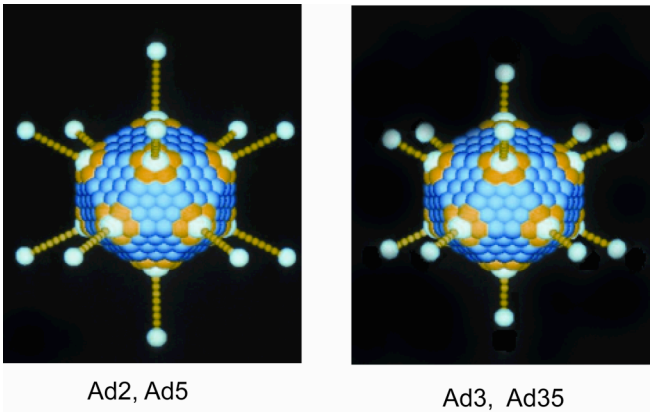

Figure 2. Structure of Ad2, Ad5 with long fibers and Ad3, Ad35 with short fibers.

Endocytic pathways used by Adenovirus Ad2 and Ad5

Ad2 and Ad5 infect respiratory epithelial cells. First, they bind to the primary receptor, Coxsackie virus B Adenovirus Receptor (CAR). Subsequent binding to secondary integrin coreceptors (e.g. alpha v beta 3 and alpha v beta 5 integrins) causes fiber shedding (Nakano et al., 2000) and triggers a variety of signalling responses including PKA and MAPK activations (Suomalainen et al., 2001), clathrin-mediated uptake of the viral particle and macropinocytosis (Greber 2002). Macropinosomes are ruptured and release their content (Meier et al., 2002) . It coincides with virus escape to the cytosol (Meier et al., 2002). and interferring with macropinocytosis decreases the infection (Imelli et al., 2004). Endosomal escape of Ad2/5 is very efficient and takes place within 15 min post infection. Once in the cytosol, Ad particles are transported along microtubule to the microtubule organizing center (MTOC) (Greber and Way 2006) and further to the nuclear pore complex where they bind to Can/NUP214 and release their DNA together with associated proteins V and VII (Greber et al., 1993, Strunze et al., 2005 and Trotman et al., 2001).

CD46, the receptor of Species B Adenovirus

All Ads bind CAR except the Species B Ads which bind CD46 for primary attachment (Sirena et al., 2004; Segerman et al., 2003; Gaggar et al., 2003 and Marttila et al., 2005). Another exception is the Species D Ad37 that binds to either sialic acid and/or

to some extent CD46 (Arnberg et al., 2000; Wu et al., 2004). CD46 (also called membrane cofactor protein MCP) is expressed on all nucleated cells of human origin. Initially it was identified as a factor binding and inactivating C3b and C4b complement products, a process that protects human cells from lysis by autologous complement (Liszewski et al., 1991, Cattaneo, 2004). CD46 is a target of other pathogens besides Ads, such as Measles virus (Edmonton strain), human herpesvirus 6, and bacteria like Streptococcus pyogenes and pathogenic Neisseria (Cattaneo, 2004; Okada et al., 1995; Kallstrom et al., 1997). The surface exposed structure of CD46 mainly consists of 4 so-called complement control protein repeat (CCP 1 to 4) from which CCP1, 2 and 4 are N-glycosylated, followed by a highly O-glycosylated serine-threonine-proline rich domain that exists in two different splicing forms, BC or C. The hydrophobic transmembrane region is followed by charged cytoplasmic tails. Due to alternative splicing of the CD46 mRNA, there are two non-homologous cytoplasmic tails of a length of either 16 or 23 amino acids that contain signaling motifs (Cyt1 and Cyt2; Fig. 3). Additional alternative splicing gives rise to 4 different splicing forms, termed BC1 and BC2 as well as C1 and C2 (BC for the STP-domains and 1 or 2 for the cytosolic tail) which are expressed in similar ratio on most cell types (Riley-Vargas et al., 2004; Russell 2004). In cancer cells there is also another splice form ABC expressed, with an additional serine-threonine-prolince rich domain termed A (Liszewski et al., 1991).

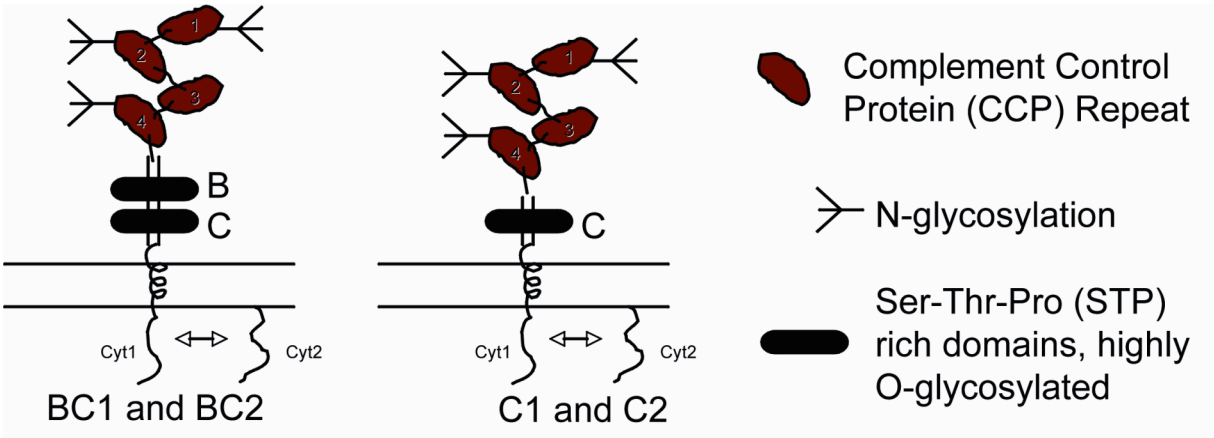

Figure 3. Model of CD46 with 4 splice forms BC1, BC2 and C1, C2. The splice form ABC that is expressed only in cancer cells is not shown.

Binding site of Species B Ad35 was determined by different assays, e.g. mutational analysis or specific antibody competition experiments. It is localized to the two

outermost CCP repeats 1 and 2 (Fleischli, et al., 2005; Gaggar et al., 2005). Further studies including Ad3, Ad7 and Ad11 revealed that all Species B Ads engage CD46 through similar binding surfaces (Fleischli et al., 2007).
Although the cytoplasmic tails of CD46 carry signaling motifs a deletion caused no drop in infection with Ad35 indicating that additional coreceptors or factors are needed for the proper infection (Sakurai et al., 2005).

Endocytic pathways and intracellular trafficking of Species B Adenovirus

In comparison to Species C Ad serotype 5 that rapidly escape from endocytic compartment and are targeted to the nucleus, Species B Ad7 accumulates within endocytic organelles and is apparently less efficiently transported towards the nucleus in certain cell types (Defer et al., 1990). The endocytosis per se was as efficient with both Ad5 and Ad7 (Miyazawa et al., 1999). Using carboxyfluorescein labeled virions the pH of the immediate environment was determined. Whereas Ad5 was not exposed to a pH lower than 6 within the first hour of infection, Ad7 accumulated in a compartment with a pH lower than 5.5. (Miyazawa et al., 2001). Partial colocalization with late endosomal/lysosomal markers have further suggested that the nature of this compartment is acidic, but it remainded unknown if this compartment was required for infection or not. It was concluded that Species B Ads escape to the cytosol out of late endosomes/lysosomes, without showing that the virions in the low pH environment are leading to infection.

The nature of the endocytosis machinery involved in Species B Ad7 entry by e.g. specific drugs or overexpression of dominant-negative constructs was not studied in detail. However, both Ad35 and Ad3 revealed similar intracellular trafficking pattern like Ad7 (Shayakhmetov et al., 2003 and Defer et al., 1990).

Use of Ads as Vectors

Adenovirus belongs to the most promising candidates among viruses for gene and cancer therapy, due to their efficient gene delivery. One drawback of the best studied Species C Adenovirus Ad2 and Ad5 is their receptor usage: CAR, the primary receptor of most of the human Adenoviruses, is low abundant in many different cell

lines, e.g. blood cells. Infection occurs only using high doses of viruses. Additionally the expression of foreign genes are only short-term, as the genomes are not integrated into the host cell genome.
One way to overcome the problem of the receptor-usage is the use of fiber-exchanged socalled fiber-swapped viruses where either the fiber knob or the whole fiber is exchanged by a fiber knob/fiber of a Species B Ad. These Ads show different binding pattern and given that they bind CD46, they can target a broader range of cells, e.g. blood cells (Marini et al., 2000). Hematopoietic stem cells as an example, were not considered as good adenoviral targets using Ad2 or Ad5 derived vectors but become more interesting targets when applying fiber-swapped viruses or Species B wild-type viruses (Marini et al., 2002).

Little is known how fiber-swapped viruses enter the host cell, but it is likely that a different route into cells is taken by fiber swapped virus in comparison to the wildtype. It was shown for a Ad5/F7 with the fiber knob of Ad5 replaced by a knob of Ad7 that it takes an Ad5 different internalization route, similar to Ad7 (Miyazawa et al., 1997). The same was true by another study using the fiber knob of Ad35 (Shayakmetov et al., 2000). Here, Ad5/F35 was shown to be retentioned in late endosomes/lysosomes, a difference to the wildtype Ad5. However, a more detailed investigation of Species B Adenovirus (wildtype and fiberswapped virus) is needed.

Additionally human Species B2 Ads Ad11 and Ad35 are rare in the human population resulting in a low seroprevalence. This is an additional important fact to investigate these viruses since vector applications derived from Ad11 or Ad35 might be more effective in comparison to e.g. Ad2/Ad5 derived vectors (Stone and Lieber, 2006).

A recent study summarized the latest results of clinical trials on different cancer using a recombinant adenovirus expressing the tumor suppressor gene p53 (rAd-p53, Peng, 2005). rAd-p53 is a virus that is not able to insert the genome into cellular chromosomes and it is replication incompetent since the p53 gene was inserted into the E1B region that is needed for replication. This rAd-p53 was injected intratumorally into head and neck squamos cell carcinoma and applied as single treatment or in combination with anti-cancer drugs like cis-platin. It has been shown that the vector applied in this study was safe since next to a few patient showing fewer no side effect

were determined. In comparison to patient receiving rAdp53 where 5 years after treatment no relapse was monitored, the patient receiving only surgery showed after 3 years a relapse rate of 30% (Peng, 2005). This is very promising for future investigations.

In vivo Ad infection

Mouse model either expressing human CAR or human CD46 were generated to study human adenovirus infections in vivo. The CAR mouse model was produced since CAR is not expressed on all cells, therefore a transgene mouse was made, expressing a truncated version with the extracellular domains of human CAR. In all analyzed organs this form of CAR was expressed (Tallone et al., 2001). In this system, in vivo administration of Ad vectors resulted in infections of macrophages, lymphocytes and endothelial cells. Furthermore, tail vein injection revealed targeting of virus into lung and capillaries of the brain, areas that were inaccessible with Ads before (Tallone et al., 2001).

Species B Ad derived vectors like Ad35 were shown not to mediate efficient transduction in mouse organs, very likely because the mouse analog of human CD46 is limited to the testis. Therefore transgenic mice were produced, that express human CD46 in all organs (Sakurai et al., 2006; Yannoutsos et al., 1996). It has been demonstrated that in human-CD46 mice recombinant Ad35 vectors were sequestred to the lungs and not, as Ad5 derived vectors did in these animals, in the liver (Verhaagh et al., 2006).

Aim of the thesis

The species B adenoviruses comprise a growing number of human pathogens with a potential in medical therapies, like gene therapy or cancer research. Recently a prevalent receptor of species B Ads has been characterized, CD46, and shown that it is expressed on all nucleated human cell lines.

The goal of this thesis was to elucidate the entry and infection route of Species B human adenovirus Ad3 and Ad35 into host cells. As model systems, epithelial HeLa and A549 cells, as well as K562, a cell line of hematopoietic origin were used. Although both viruses bind to CD46 and infect CD34 positive stem cells, the tropism of the two Ads is different, species B1 Ad3 infecting respiratory organs and blood cells, whereas species B2 Ad35 infects urinary tract and the kidneys. It is unknown which steps of infection after primary receptor binding are causing this difference.

Since species C Ads bind to integrin coreceptors after primary receptor binding, and species B Ads expose a similar RGD motif in their penton base that might bind to integrins a possible involvement of integrins in Species B Ad infection is tested. Further, stimulation of macropinocytosis is tested, since this pathway plays an important role in species C Ad infection and the signalling following the induction of macropinocytosis is investigated.

Subversion of CtBP1 controlled macropinocytosis by human Adenovirus serotype 3

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Figure 3 from Stefan Kälin
Figure 8B from Jason Mercer and Beat Amstutz

Abstract

Endocytosis supports cell communication, growth, and pathogen infection. The species B human adenovirus serotype 3 (Ad3) is associated with epidemic conjunctivitis, and fatal respiratory and systemic disease. Here we show that Ad3 uses dynamin-independent endocytosis for rapid infectious entry into epithelial and hematopoietic cells. Unlike Ad5 which uses dynamin-dependent endocytosis, Ad3 endocytosis spatially and temporally coincided with enhanced fluid phase uptake. It was sensitive to macropinocytosis inhibitors targeting F-actin, protein kinase C, the sodium-proton exchanger, and Rac1 but not Cdc42. Infectious Ad3 macropinocytosis required viral activation of p21-activated kinase 1 (PAK1), and the C-terminal adenovirus E1A binding protein 1 (CtBP1), recruited to macropinosomes. These macropinosomes also contained the Ad3 receptors CD46 and alpha v integrins. CtBP1 is a phosphorylation target of PAK1, and bifunctionally involved in membrane traffic and transcriptional repression of cell cycle, cancer and innate immunity pathways. Phosphorylation defective S147A-CtBP1 blocked Ad3 but not Ad5 infection, providing a direct link between PAK1 and CtBP1. The data show that viruses induce macropinocytosis for infectious entry, a pathway used in antigen presentation and cell migration.

Introduction

Animal cells support a variety of endocytic pathways to coordinate signal transduction, cell growth, differentiation, death, and also pathogen infection (Marsh and Helenius, 2006). Besides clathrin-mediated endocytosis, dynamin-dependent endocytic pathways include phagocytosis, the interleukin-2 receptor and intercellular adhesion molecule 1 routes, caveolar and lipid raft-dependent uptake, or dorsal ruffling leading to WAVE complex-dependent endocytosis (Gruenberg and van der Goot, 2006; Marsh and Helenius, 2006; Orth et al., 2006). Only a few dynamin-independent pathways are known, e.g., cholera toxin uptake via the glycosylphosphatidylinositol (GPI) anchored protein pathway (Kirkham et al., 2005), or macropinocytosis which engulfs large plasma membrane domains into spherical endosomes (Schnatwinkel et al., 2004). Macropinocytosis can be induced by growth factors, Ras or Src tyrosine kinase. It clears bacterial infections, removes apoptotic bodies, and is an important element of innate immunity and cell migration (Wu et al., 2006). Constitutive macropinocytosis supports antigen processing and presentation in dendritic cells. In viral infections, macropinocytosis is an accessory pathway triggered by HIV (Liu et al., 2002; Marechal et al., 2001), and human adenovirus types 2 and 5 (Ad2/5) in respiratory epithelial cells (Meier et al., 2002). It has remained unclear, however, if macropinocytosis is an infectious entry pathway for pathogens.

Adenoviruses are a diverse family of agents. They infect the upper and lower respiratory tracts, the urinary and digestive tracts, and give rise to epidemic conjunctivitis. The species B serotypes Ad3, Ad7 and Ad11 are associated with exacerbations of asthmatic conditions, and mobidity and mortality, and Ad3 has been associated with epidemic conjunctivitis (Hayashi and Hogg, 2007). The nature of the Ad3 receptor has been debated (Marttila et al., 2005; Sirena et al., 2004), but recent mutagenesis experiments have mapped the Ad3 binding site of CD46 to the distal consensus repeats 1 and 2 (Fleischli et al., 2007), in close agreement with the crystal structure of the Ad11 fiber knob on CD46 (Persson et al., 2007). CD46

protects autologous cells from complement attack, and links innate and acquired immunity in macrophages and lymphocytes (Liszewski and Atkinson, 1996; Riley-Vargas et al., 2004). The extracellular domain contains partly overlapping binding sites for the complement factors C3b and C4b, and different viruses and bacteria. In T-cells, ligand-induced crosslinking of CD46 stimulates the phosphorylation of Cbl and LAT, and the activation of Vav, Rac, and the Erk1/2 MAP kinase pathway. The Rac1 GTPase orchestrates CD46 downregulation by macropinocytosis (Crimeen-Irwin et al., 2003), and increases susceptibility to complement-mediated lysis, and immune suppression (Kemper et al., 2005).

Here we show that infectious endocytic uptake of Ad3 occurs through macropinocytosis. It is controlled by a transcriptional corepressor, the C-terminal binding protein 1 of E1A (CtBP1). CtBP1 occurs in two spliced forms, long and short (CtBP1-L/S). CtBP1-S (also called brefeldin A-ribosylated substrate, BARS) lacks 11 N-terminal amino acids, and like CtBP1-L is involved in dynamin-independent endocytosis (Bonazzi et al., 2005; Chinnadurai, 2002). It is observed at Golgi membranes, and functions in mitotic partitioning of the Golgi apparatus and membrane trafficking (Colanzi et al., 2007). It is also found on dense bodies of synaptic termini where it may have a tethering role in membrane organization (Gallop et al., 2005; tom Dieck et al., 2005). The two mammalian CtBP genes 1 and 2 have partly overlapping transcriptional functions in tumorigenesis, apoptosis, development, cell differentiation, cell cycle regulation, and viral infection (Berk, 2005). In normal cells, CtBP1 heterodimerizes with CtBP2, and shuttles between the nucleus and the cytoplasm depending on posttranslational modifications and binding to PDZ proteins (Barnes et al., 2003; Verger et al., 2006). Here we address a cytoplasmic function of CtBP1 in infectious macropinocytic uptake of Ad3.

Results

Alpha v integrins in dynamin-dependent and -independent Ad3 endocytosis

Integrins have been known to support infectious endocytosis of species C human adenoviruses, such as respiratory Ad2 and Ad5 (Stewart and Nemerow, 2007). We tested if Ad3 infection required alpha v integrins. Ad3-eGFP transduction of alpha v integrin deficient, CD46 positive M21L melanoma cells was reduced compared to alpha v integrin positive M21 cells, although binding was not affected (Fig. 1A, B), in agreement with earlier reports on infection (Mathias et al., 1994). Similar results were obtained with Ad5 (Fig. 1B). Soluble RGD peptides inhibited both Ad3 endocytosis measured by trypsin resistance of (3H)-thymidine labeled virions (Fig. 1C), and transduction of Ad3-eGFP (Fig. 1D). Quantitative internalization assays at low multiplicity of infection (moi, 5 infectious particles bound per cell) indicated that more than 50% of the surface bound virions were internalized within 20 min (Fig. 1E), consistent with virus uptake measurements at high moi (Fig. 2A). This result was similar for Ad2 (Greber et al., 1993), and the Ad3-related Ad7 (Miyazawa et al., 1999). Ad3-eGFP transduction of HeLa-ATCC cells was not sensitive to dominant-negative (dn) K44A dynamin2 (dyn2), which blocked transferrin uptake, whereas Ad5 was strongly inhibited by K44A-dyn2 (Fig. 1F, G). In HeLa-K cells (a variant of HeLa-ATCC), K44A-dyn2 inhibited both Ad3 and Ad5-mediated eGFP transduction, although Ad5 was affected more strongly, and transferrin uptake was inhibited similarly as in HeLa-ATCC cells (Fig. 1H, I). To further assess an involvement of dynamin-mediated endocytosis in Ad3 infection we expressed dn constructs of amphiphysin and Eps15, and measured nuclear targeting of fluorescent Ad3 (Nakano and Greber, 2000). The D36R mutant of the amphiphysin2 SH3 domain which tightly binds to and prevents self assembly of dynamin (Owen et al., 1998), and the Eps15 EH29 mutant lacking EH domain 2 but retaining binding to the adaptor complex 2 (Benmerah et al., 1999) blocked transferrin uptake but not Ad3 nuclear targeting, indicating that dynamin and Eps15 were not involved in Ad3 endocytosis in HeLa-ATCC (Fig. 3). This suggested a cell type-dependent, but low

level involvement of dynamin in Ad3 infection, and confirms a strong dynamin requirement for Ad5 infection.

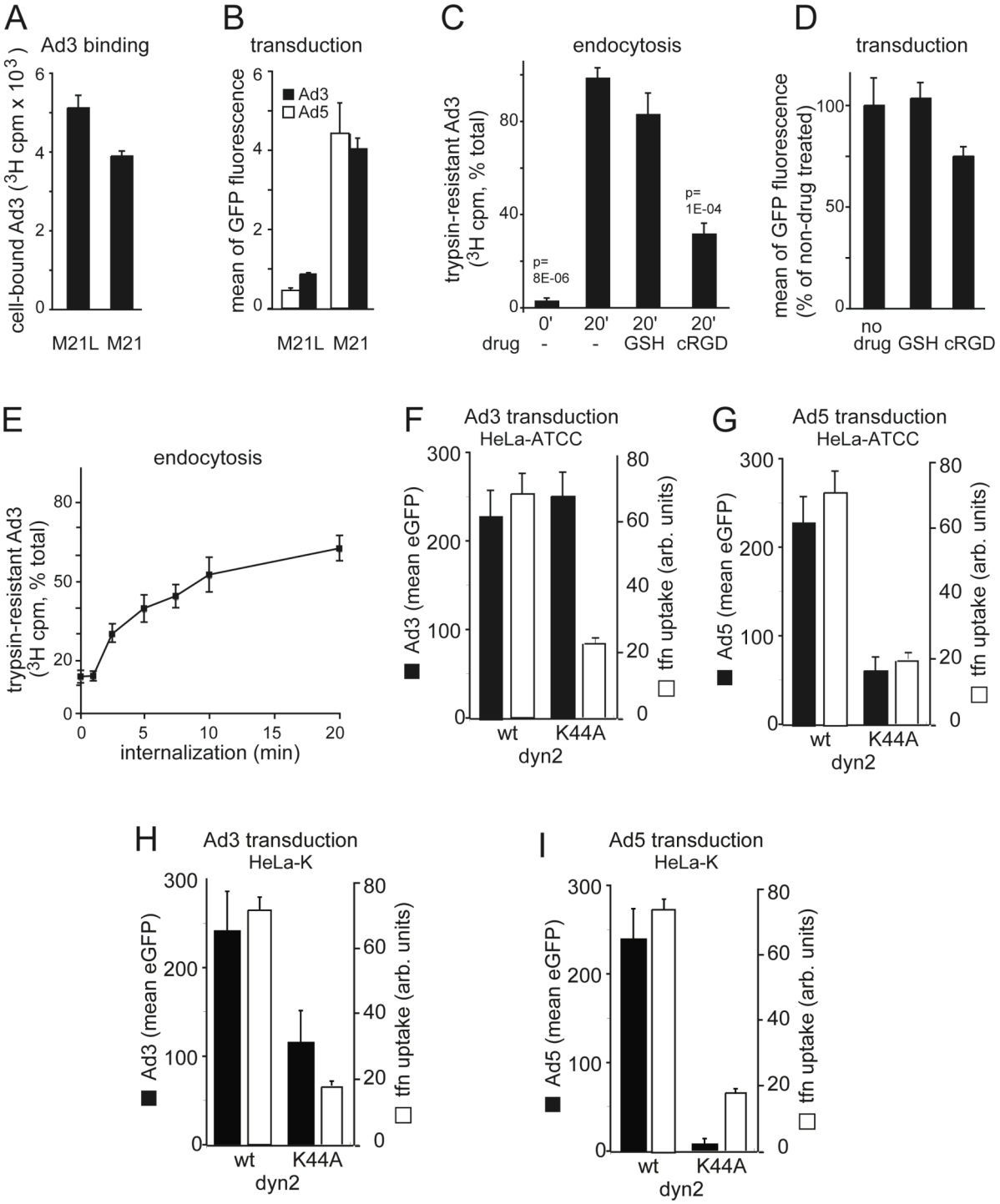

Fig. 1: Infectious Ad3 endocytosis of HeLa cells requires alpha v integrins, and to a low extent dynamin.

A: Human melanoma M21 or M21L cells were incubated with (3H)-labeled Ad3 in the cold, and analyzed for cell-associated radioactivity (10^6 cells, 0.75 µg Ad3). B: M21 or M21L cells were transduced with Ad3-eGFP or Ad5-eGFP (moi 5) and analyzed by flow cytometry 8 hrs p.i.. C, D: (3H)-labeled Ad3 (8 X 10^5/ml; 50000 cpm) was cold bound to HeLa-ATCC cells (2000 cpm bound),

and warmed for 0 or 20 min in the presence or absence of gluthathione (GSH) or cyclic RGD peptide (cRGD, 0.2 mM), trypsinized in the cold, and analyzed for cell-associated (internalized) or released (3H)-Ad3 by liquid scintillation counting. Cells were treated with GSH or cRGD, infected with Ad3-eGFP (moi 5), and analyzed for GFP expression by flow cytometry 6 h p.i.. E: Kinetics of Ad3 endocytosis measured by trypsin resistance as described in panel C (100% equivalent to 2000 cpm). Means of triplicate dishes, one representative experiment (A-E). F,G,H,I: Ad3-eGFP and Ad5-eGFP transduction (6 h) and transferrin-647 internalization (10 µg/ml transferrin in the last 30 min of infection, open bars) in HeLa-ATCC or HeLa-K cells transfected with wt dynamin2 (wt dyn2) or K44A dyn2 for 48 h. Single cell analysis by confocal microscopy (moi 5), showing the mean of at least 40 blindly selected cells. One representative experiment is shown (F-I).

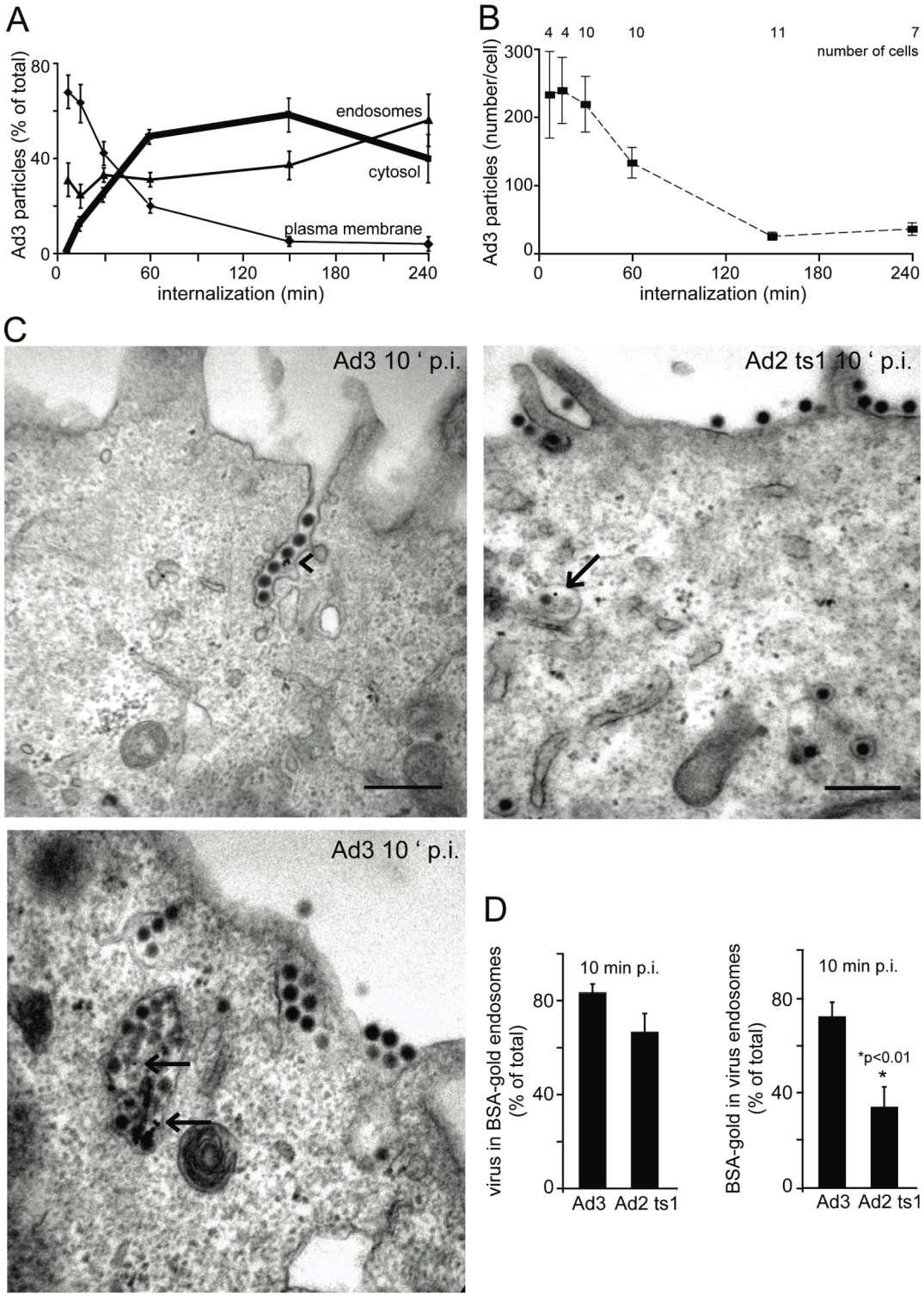

Fig. 2: Quantitative EM analyses of Ad3 endocytosis and endosomal escape.

A: Distribution of cold-bound Ad3 (5 x 10^5 viral particles per cell, 4°C, 60 min) on the plasma membrane, endosomes and the cytosol (bold line) upon internalization at 37°C. B: Analyses of the total number of particles and cells. C,D: Enrichment of Ad3 in fluid phase positive endosomes. HeLa cells were incubated with Ad3 or Ad2-ts1 in the cold (moi as in A), washed, pulsed with BSA-gold for 10 min, fixed for ultra thin section EM analyses, and quantified for viral particles in either gold-positive endosomes (arrows), or gold particles in endosomes that contain Ad3 or Ad2-ts1, respectively. Viruses in plasma membrane invaginations are pointed out by arrow head.

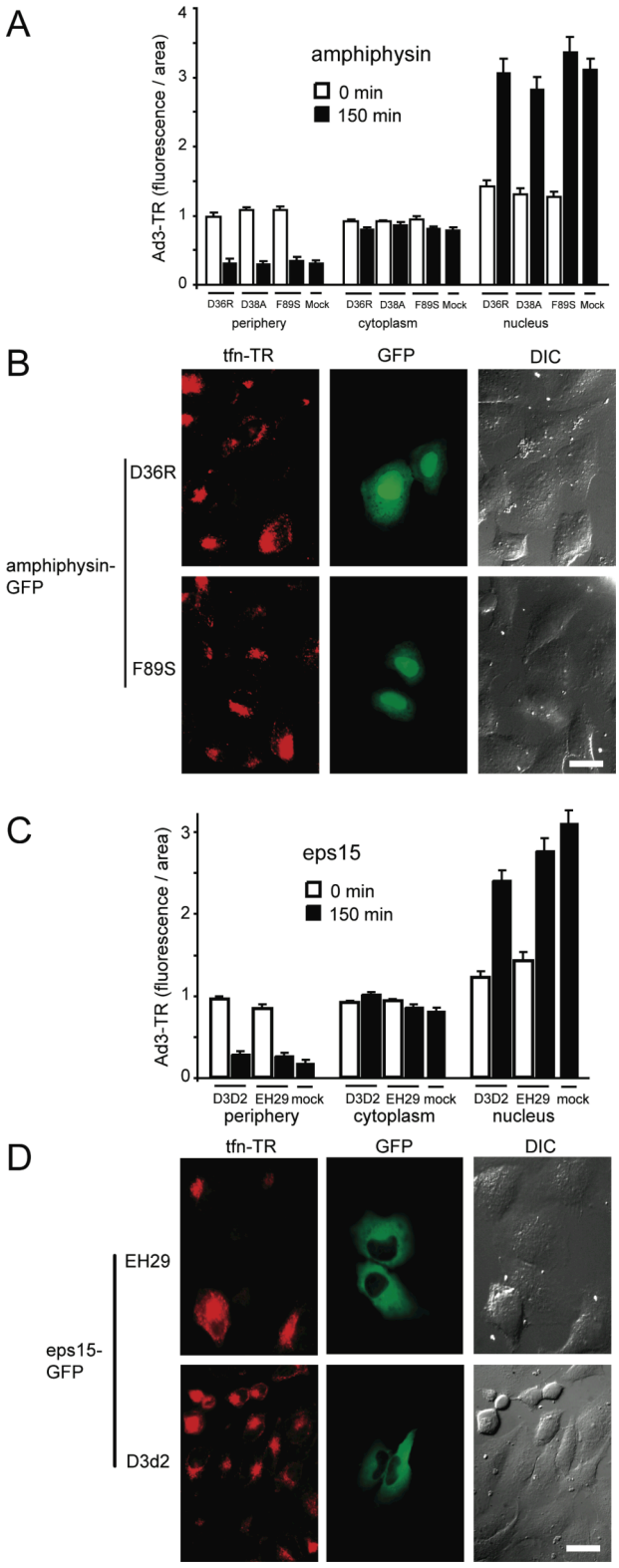

Fig. 3: Ad3-TR nuclear targeting and Ad3-eGFP transduction are independent of amphiphysin.

A: Ad3-TR (0.5 µg/ml) was cold bound to HeLa cells transfected with D36R amphiphysin2 SH3 domain (24 h). This mutant protein binds very tightly to dynamin, and blocks transferrin uptake, while D38A or F89S amphiphysin2 SH3 domains which do not bind dynamin did not affect transferrin uptake (Owen et al., 1998). None of the amphiphysin constructs affected Ad3-TR targeting to the nucleus 150 min p.i.. measured by subcellular localization of fluorescent virus particles (Nakano and Greber, 2000). B: D36R potently blocked the uptake of transferrin labeled with texas red, as expected. C: Ad3-TR (0.5 µg/ml) was cold bound to HeLa cells transfected with EHdelta2 Eps15, which blocks transferrin uptake, or D3D2 Eps15 which does not affect transferrin uptake (Benmerah et al., 1999), and internalized for 150 min. D: EHdelta2 Eps15 but not D3D2 Eps15 blocked the uptake of fluorescent transferrin.

Macropinocytosis is an infectious entry route of Ad3

EM analyses of incoming Ad3 at high moi of 5000 confirmed that the bulk of Ad3 was internalized rapidely and efficiently (Fig. 2A). Viral escape to the cytosol occurred with a half time of 30 to 40 min pi. At 150 min pi, 90% of the particles morphologically disappeared, indicating capsid disassembly and / or degradation (Fig. 2B). Ad3 particles were frequently found in smooth invaginations of the plasma membrane, and large vesicles with multiple virus particles (Fig. 2C, 10 min pi). The limiting membrane of viral vesicles was not stained with cell impermeable cationized ferritin added after virus internalization indicating that these structures were in the cytosol (not shown). About 80% of the Ad3 carrying vesicles contained fluid phase BSA-gold, similar to vesicles with Ad2-ts1 (Fig. 2D). Ad2-ts1 is internalized in the absence of macropinocytosis with similar kinetics as wild type Ad2 (Meier et al., 2002). In contrast to Ad2-ts1, Ad3 endosomes contained more BSA-gold demonstrating that Ad3 enters along a pathway rich in fluid phase (Fig. 2D). Dextran measurements confirmed that Ad3 stimulated fluid uptake (Fig. 4A, B). Ad3 but not Ad2-stimulated dextran uptake was completely abolished in the presence of excess Ad3 fiber knob protein which binds CD46 and blocks cell attachment of Ad3 (Sirena et al., 2004) (Fig. 4C). The fiber knob of the Ad3-related Ad11 is a trimer that binds three soluble CD46 extracellular consensus repeats (Persson et al., 2007). The data thus suggested that multiple fibers or additional capsid components of the Ad3 particle are required to trigger CD46-mediated macropinocytosis.

To test if antibody-mediated CD46 crosslinking triggered fluid phase uptake, HeLa-ATCC cells were incubated with the monoclonal anti-CD46 IgG antibody E4-3, followed by anti-IgG crosslinking antibodies. Under these conditions, we measured

a modest but significant 1.6 fold increase of dextran uptake compared to control cells or cells incubated with 5x lower concentrations of E4-3 (Fig. 4D). This was in agreement with an earlier study reporting that low antibody crosslinking of CD46 leads to CD46 internalization by constitutive clathrin-mediated endocytosis, whereas a high degree of crosslinking leads to cell surface ruffling and macropinocytosis (Crimeen-Irwin et al., 2003). Cells incubated with the anti-alpha v integrin antibody LM609 but not the function blocking P1F6 stimulated dextran uptake 1.5 fold (Fig 4D). The combination of E4.3 and LM609 gave a robust 2-fold stimulation of dextran uptake suggesting that simultaneous CD46 and alpha v integrin crosslinking potentiates fluid phase uptake. Ad3 alone was more potent than antibodies or the phorbol ester PMA, and depended on soluble cyclic RGD (arginine-glycin-aspartate) peptides (Fig. 4D, E). We concluded that Ad3 stimulates fluid phase uptake by the combined ligation of CD46 and alpha v integrins.

We next assessed Ad3-stimulated dextran uptake by pharmacological inhibitors. Ad3-stimulated dextran uptake was sensitive to cytochalasin D and jasplakinolide, which depolymerize and stabilize F-actin, respectively, and the protein kinase C inhibitor Gö6976, which blocks the calcium-dependent PKC alpha and beta isoforms but not calcium-independent PKCs (Davies et al., 2000; Gschwendt et al., 1996), and strongly blocked by the sodium/proton exchange inhibitor 5-(N-ethyl-n-isopropyl)-amiloride (EIPA) (Fig. 4F, G). Importantly, all these classical macropinocytosis inhibitors significantly reduced Ad3 infection, although jasplakinolide could also have post-endocytic effects inhibiting Ad3-eGFP expression, as concluded by the late addition of the drug (Fig. 4H). These inhbitors as well as the Rho GTPase inhibitor toxin B prevented viral endocytosis measured at low moi using the trypsin assay (Fig. 5A). The analysis of subcellular localization of Ad3 particles at the plasma membrane, endosomes and the cytosol using quantitative thin section EM at high moi confirmed these results (Fig. 5B, and Fig. 7). Interestingly, both Ad3-stimulated dextran uptake and infection were also inhibited in cells depleted of clathrin heavy chain (CHC, Fig. 5C, D). Infection of CHC-depleted cells with SV40 which enters by clathrin independent endocytosis (Marsh and Helenius, 2006) was not affected (not shown). Importantly, CHC

depletion did not affect the expression of CD46, coxsackievirus adenovirus receptor (CAR) or alpha v integrins on the cell surface (not shown). It also did not affect basal dextran uptake which occurs by clathrin-dependent and independent endocytosis, but blocked transferrin internalization (not shown). This could suggest that another endocytic process which is different from viral macropinocytosis could compensate for the loss of clathrin-mediated endocytosis, as previously suggested for dynamin-inhibited cells which upregulate an unknown dynamin-independent pathway (Damke et al., 1994). Importantly, most of the Ad3 particles were found in large endocytic vesicles, and only a minor fraction in clathrin-coated invaginations or vesicles (less than 1%, for representative EM images see Fig. 6), suggesting that clathrin has a minor role in Ad3 endocytosis. This was confirmed by siRNA knock down of CHC and quantitative EM analyses showing that CHC knock down reduced cytosolic Ad3 but not plasma membrane or endosomal Ad3 (Fig. 5E). This shows that at high moi, Ad3 can internalize by a clathrin-independent mechanism in the absence of fluid phase stimulation. It is likely that clathrin is required for endosomal escape of Ad3, and facilitates dextran uptake, possibly involving cytoplasmic membrane transport processes, such as retromer-mediated sorting from endosomes to the Golgi (Popoff et al., 2007).

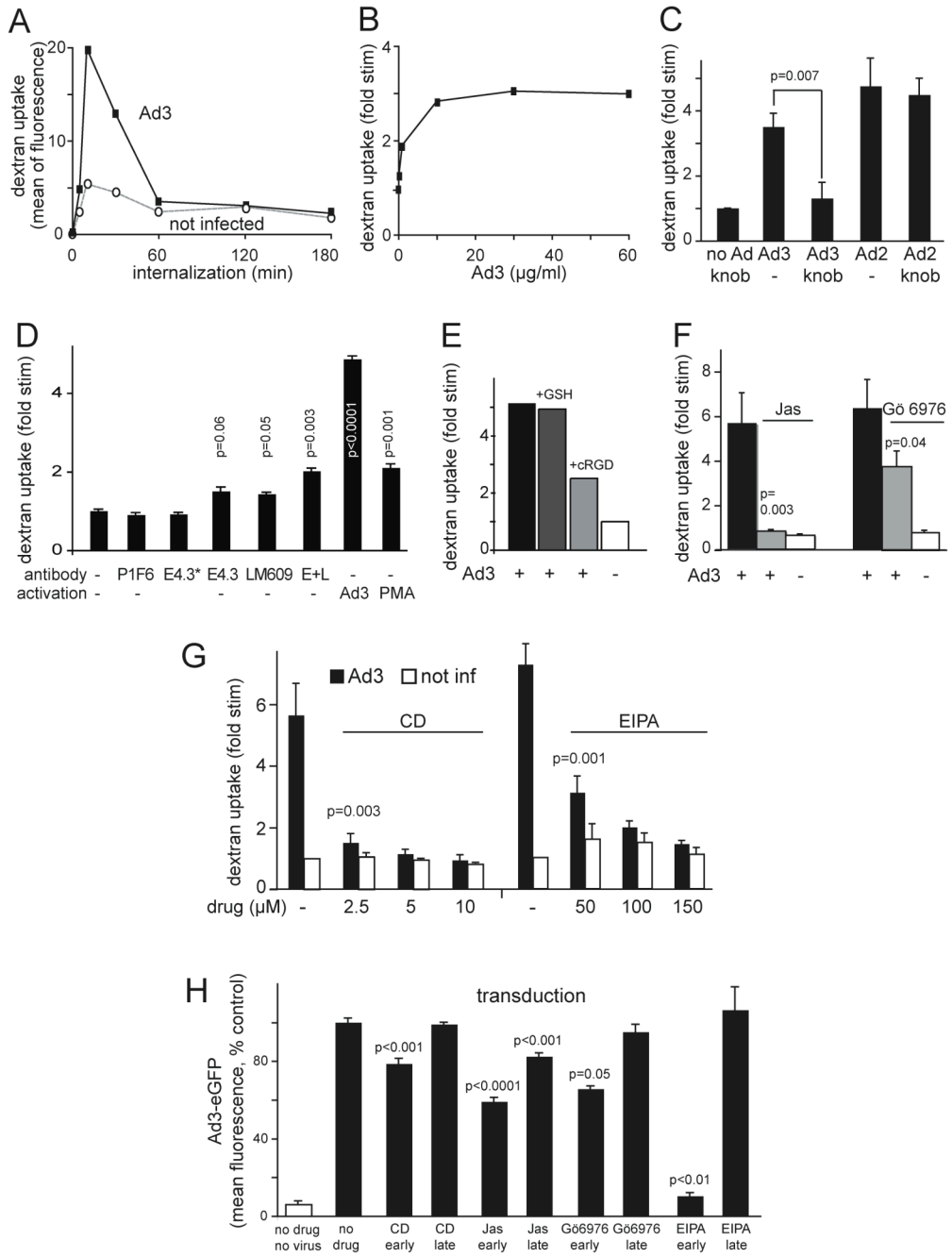

Fig 4: CD46, integrin, F-actin, PKC and EIPA dependent stimulation of fluid phase endocytosis by Ad3.

A: Ad3 transiently stimulates dextran uptake. HeLa-ATCC cells were incubated with Ad3 (5 µg/ml, equivalent to 2000 particles bound per cell) in the cold for 60 min, or noninfected, warmed for 0, 5, 10, 30, 60, 120 or 180 min, pulsed with dextran-FITC and analyzed by flow cytometry as described in Materials and Methods. One out of three representative experiments is shown. B: Dose dependence of fluid phase uptake stimulation expressed as fold stimulation over noninfected cells. HeLa cells were incubated with different amounts of Ad3 (0, 0.5, 1, 10, 30, 60 µg/ml) in the cold, warmed for 5 min, and pulsed with dextran-FITC for 5 min, washed and analyzed by flow cytometry. One out of two similar experiments is shown. C: Specificity of Ad3 stimulated fluid phase endocytosis. HeLa cells were incubated with Ad3 or Ad2 (5 µg/ml) in the presence or absence of Ad3 fiber knob (5 µg/ml), and analyzed for dextran-FITC stimulation as described above. D: Fluid phase stimulation by anti-CD46 and anti-integrin antibodies. HeLa cells were incubated with 4 µg/ml anti-CD46 antibody E4.3, or 0.8 µg/ml E4.3 (E4.3*), anti-alpha v beta 5 integrin (P1F6), anti-alpha v beta 3 (LM609) or a combination of E4.3 plus LM609 (E+L, 4 µg/ml each) in cold RPMI medium for 1h, washed and

incubated with goat anti-mouse IgG antibodies (10 µg/ml), Ad3 or the phorbol ester PMA (Meier et al., 2002) on ice for 30 min, warmed in the presence of dextran-FITC for 10 min and analyzed by flow cytometry. The experiment was performed in triplicate and repeated once. E,F,G: Measurement of Ad3 induced dextran-FITC uptake 10 min p.i. in the presence or absence of cyclic RGD peptides (0.1 mM, Meier et al., 2002), jasplakinolide (40 nM Jas), the PKC inhibitor Gö6976 (1 µM), or various concentrations of cytochalasin D (CD) or N,N-ethyl,-isopropylamiloride (EIPA). H: Ad3-eGFP transduction (1000 viral particles/cell) of HeLa cells pretreated with CD, Jas, Gö6976, or EIPA. Gö6976 and EIPA were present during 1h warm infection, and were then washed off (early), or present till 120 to 180 min p.i. (late), followed by flow cytometry of eGFP 6 h p.i.. CD and Jas were present during the entire incubation time (early), or added 1h post warming (late). Experiments were performed at least twice with triplicate samples.

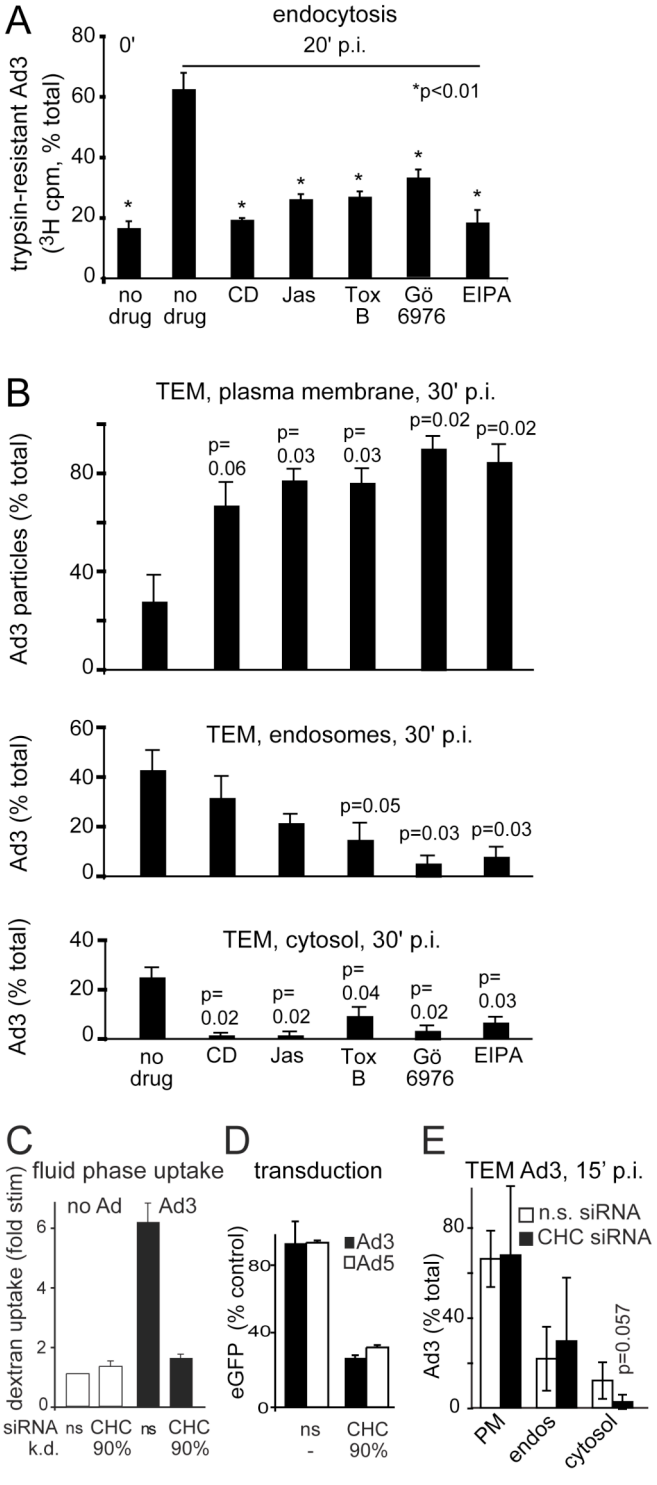

Fig. 5: Ad3 endocytosis and endosomal escape require F-actin, Rho GTPases, PKC, the sodium proton exchanger 1 and clathrin.

A: Endocytosis of Ad3 measured by trypsin sensitivity of cell surface localized virus. HeLa-ATCC cells were pretreated with cytochalasin D (CD, 5µM), Jasplakinolide (Jas, 0.3 µM), Clostridium difficile toxin B (toxB, 0.3 µg/ml) (Aktories, 1997), Gö6976 (1 µM), or N,N-ethyl,-isopropoylamiloride (EIPA, 100 µM) in growth medium for 30 min, incubated with (3H)-Ad3 (50000 cpm) in the cold for 1h, washed and internalized at 37°C for 20 min, washed with cold medium and treated with trypsin (2 mg/ml) at 4°C for 1 h. Cells were pelleted by centrifugation at 500 x g, and supernatants and cell pellets analyzed by liquid scintillation counting (fraction of total, 100% equivalent to 2000 cpm). B: Analysis of subcellular localization of Ad3 particles by transmission EM. HeLa cells were pretreated with drugs as described in A, incubated with Ad3 (30 µg/ml) in the cold, washed with binding medium, internalized in drug-containing medium for 30 min, and fixed for ultra thin section EM analyses. Viral particles were quantified at the plasma membrane, in endosomes, and the cytosol as described (Meier et al., 2005). The total number of particles blindly analyzed for each condition were 200-300 in 6-9 different cells. For representative images see Fig. 7 C,D,E: Ad3 stimulated dextran uptake and infection required CHC. Uptake of dextran-FITC in normal HeLa-ATCC cells or cells transfected with nonsilencing siRNA (ns), or siRNA against CHC (CHC, double transfection, 72 h), and eGFP transduction measurements, as well as EM analyses occurred as described.

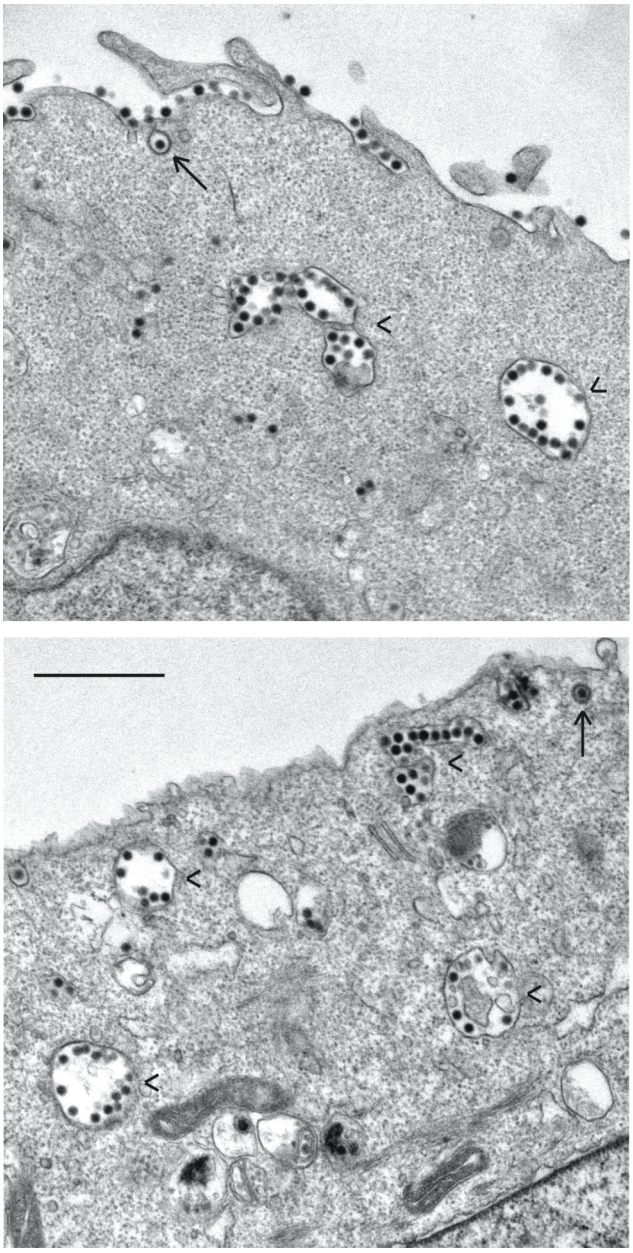

Fig. 6: Thin section EM analyses of incoming Ad3.

HeLa-ATCC cells were infected with Ad3 at high moi (30 µg/ml) for 30 min, fixed, stained and prepared for EM analyses. Virus particles in macropinosomes are indicated by arrow heads, and 2 particles in clathrin-coated vesicles are indicated by arrows. Bar = 1 µm.

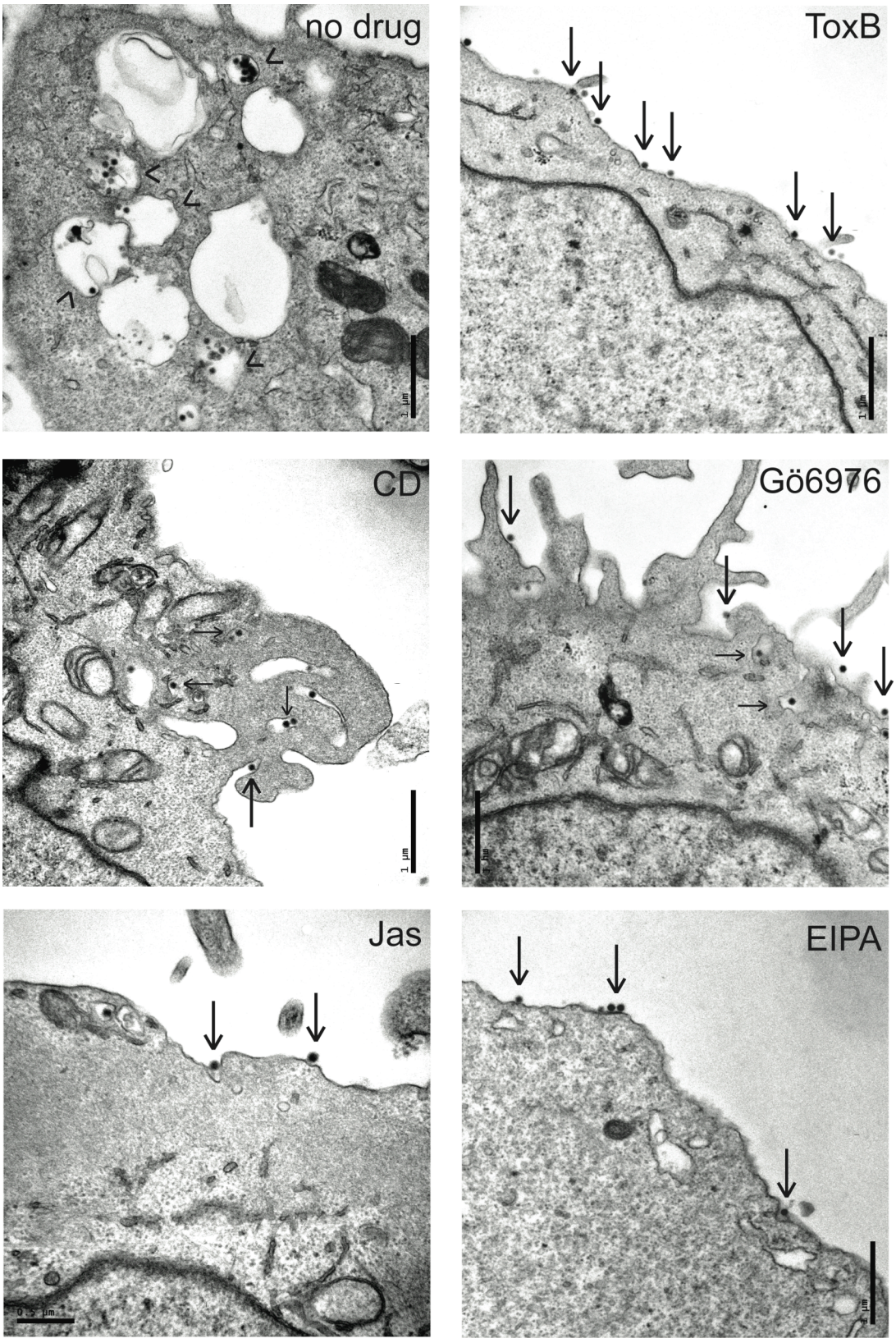

Fig. 7: Thin section EM analyses of incoming Ad3 in drug-treated cells.

HeLa-ATCC cells were treated with drugs in growth medium at 37°C for 30 min as indicated, incubated with Ad3 at high moi (30 µg/ml) in the cold for 60 min, washed and internalized in the presence of drugs for 30 min, fixed, stained and prepared for EM analyses. Virus particles in macropinosomes are indicated by arrow heads, viruses in small vesicles by small arrows, and viruses at the plasma membrane by large arrows. Bar = 1 µm, except for Jas (bar = 0.5 µm).

Infectious Ad3 macropinocytosis requires PAK1 activation and CtBP1

Dynamin-independent endocytosis of GPI-anchored proteins into early endosomal antigen 1 (EEA1) positive endosomes can occur along the GEEC pathway (GPI-anchored proteins enriched early endosomal compartment) involving Cdc42 (Mayor and Pagano, 2007). While the expression of the dn T17N-Cdc42 had no effect on Ad3 transduction, dn T17N-Rac1 blocked Ad3-eGFP expression (Fig. 8A, and Fig. 9), and dextran uptake (not shown) consistent with a role of Rac1 in macropinocytosis (Dharmawardhane et al., 2000). Nuclear targeting of fluorescent Ad3 was independent of EEA1 (knock down levels were larger than 95%, not shown), and dn caveolin-eGFP (not shown). Likewise, Ad3 uptake and infection were not affected in cells expressing the dn T27N or dominant-active Q67L Arf6 mutants (not shown, Kirkham et al., 2005). The data so far supported the model that crosslinking of CD46 through multiple Ad3 fibers leads to membrane ruffling and macropinosome formation. This process was enhanced by viral engagement with alpha v integrins, and required F-actin, protein kinase C and Rac1, and gave rise to Ad3 bearing macropinosomal vesicles, some of which had a similar morphological EM appearance as late endosomes and lysosomes. The escape of Ad3 (and the related Ad7) from endosomes approximately 30 min pi was considerably slower than the escape of Ad2 (t½ of 30 min compared to 15 min of Ad2) (Greber et al., 1993), although viral uptake rates were comparable, possibly reflecting different entry pathways of Ad2 and Ad3.

We next tested if the Rac1 target p21-activated kinase 1 (PAK1) was involved in Ad3 stimulated macropinocytosis. PAK1 has been implicated in growth factor stimulated macropinocytosis in fibroblasts, cell adhesion and motility (Dharmawardhane et al., 2000; Jaffer and Chernoff, 2002). T423-phospho-PAK1 measurements indicated that Ad3 and Ad5 but not Ad2-ts1 transiently induced

PAK1 activation up to 30 fold, albeit at slightly different rates. Ad3 stimulation peaked at 5-10 min, and Ad2 at 10-15 min p.i. (Fig. 8B). Expression of a PAK1 autoinhibitory domain or two different PAK1 siRNAs (P2, P8) inhibited Ad3 infection and dextran uptake, but not Ad5 infection or transferrin uptake which were independent of dn PAK1 (Fig. 8C, D). Similarly, Ad5 infection was reported to be independent of dominant active PAK1 in colon cancer SW480 cells (Li et al., 1998). Importantly, the PAK1 siRNAs inhibited the penetration of Ad3 to the cytosol (Fig. 7E, not shown). Collectively, the data suggest a role of PAK1 in Ad3 endocytosis and cytosolic escape but not in endocytosis of Ad5 or transferrin (Meier et al., 2002).

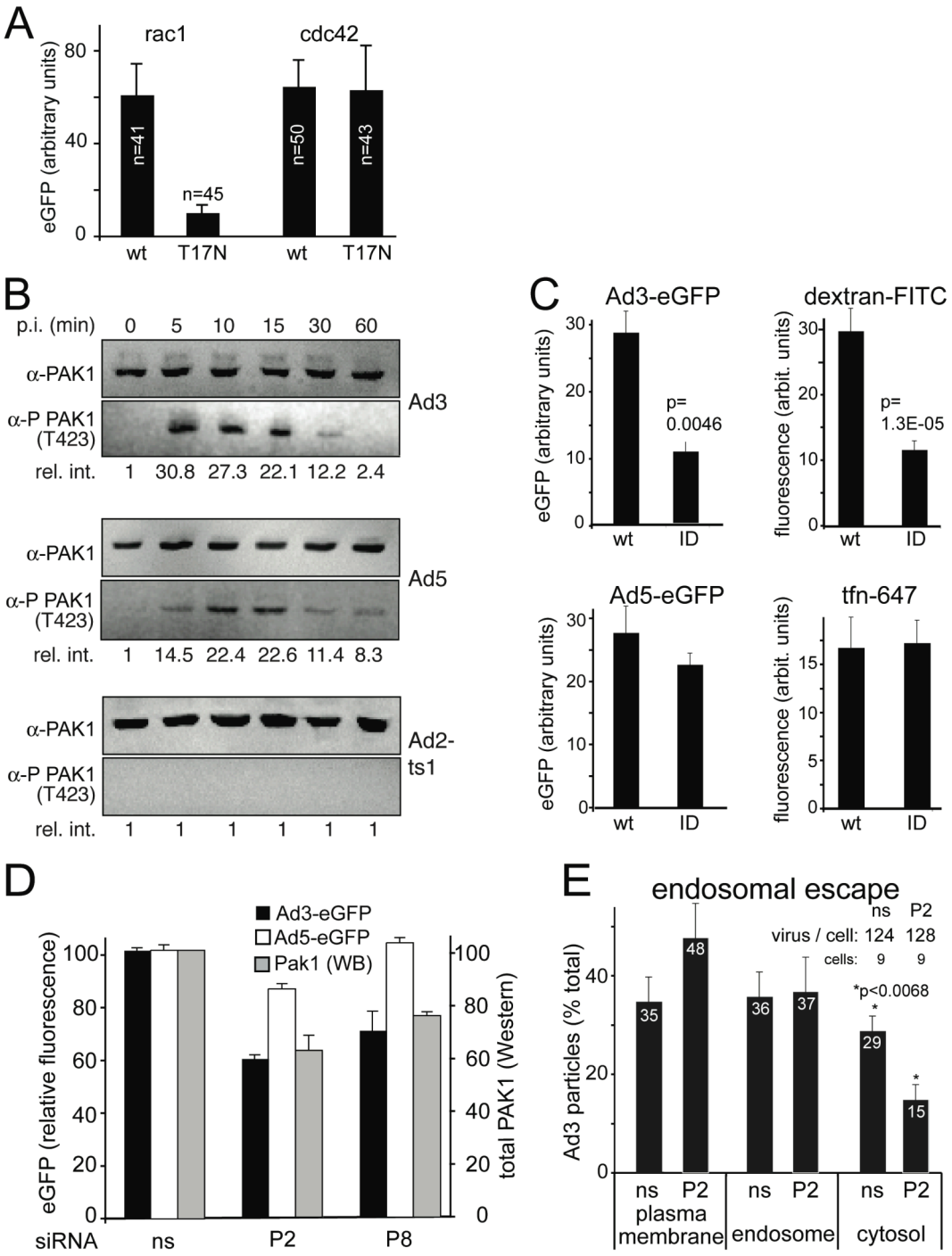

Fig. 8: Rac1 and PAK1 are required for Ad3 but not Ad5 endocytosis and infection.

A: HeLa-ATCC cells were transfected with plasmids encoding CFP-Rac1, CFP-Rac1 T17N, CFP-Cdc42 or CFP-Cdc42 T17N for 30 h, infected with Ad3-eGFP for 15 h, fixed, and analyzed by confocal laser scanning microscopy. The eGFP intensity of at least 40 CFP-positive cells per condition were quantitated by NIH image J with means and standard errors of the mean. The experiment was performed twice with similar results. Representative images are shown in Fig. 9A, B: PAK1 is activated by Ad3 and Ad5. Nonstarved HeLa-ATCC cells were incubated with Ad3, Ad5 or ts1 in the cold, washed, warmed for different times, and analyzed for PAK1 and phosphorylated PAK1 (T423) using Western blotting. One of 3 representative experiments is shown including relative intensities of quantitated phospho-PAK1 (rel int). C: Cells expressing wild type or dn PAK1 (inhibitory domain ID) were transduced with Ad3-eGFP or Ad5-eGFP, or assessed for uptake of dextran-FITC or transferrin-Alexa 647 upon Ad3 infection. D: Cells were transfected with siRNAs P2 and P8 against PAK1 or nonsilencing (ns) siRNA for 72 h (double transfection, 20 pmoles/ml siRNA),

infected with Ad3-eGFP or Ad5-eGFP for 6 h, and analyzed for eGFP expression by flow cytometry. Transfected cells (1 x 10^5) were analyzed by Western blotting (WB) for PAK1 (grey panels). E: Endosomal escape of Ad3 measured by thin section EM in HeLa cells transfected with anti-PAK1 siRNA P2 and ns siRNA, respectively. Virions were counted at the plasma membrane, in endosomes and the cytosol.

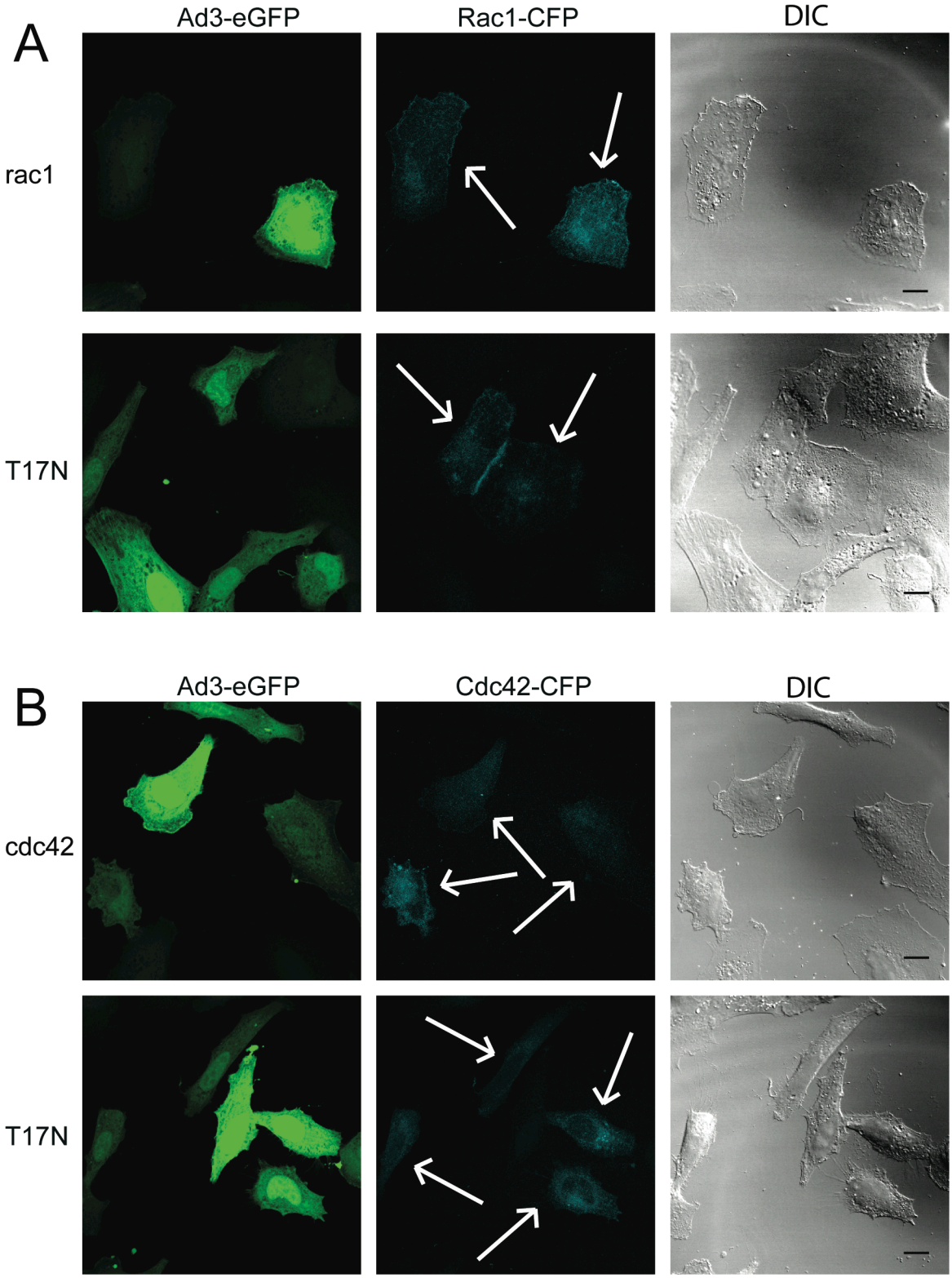

Fig. 9: Ad3-eGFP infection depends on Rac1 but not Cdc42.

HeLa-ATCC cells were transfected with plasmids encoding CFP-Rac1, CFP-Rac1 T17N (panel A), or CFP-Cdc42 and CFP-Cdc42 T17N (panel B) for 30 h, infected with Ad3-eGFP for 15 h, fixed, and

analyzed by confocal laser scanning microscopy. Transfected cells are pointed out by arrows. Bars = 10 µM.

An important effector of activated PAK1 is CtBP1 which is phosphoryted by PAK1 and recruited to the cytoplasm (Barnes et al., 2003). CtBP1 occurs in two splice forms, long and short (CtBP1-L/S). CtBP1-S (also called brefeldin A-ribosylated substrate, BARS) and CtBP1-L are involved in dynamin-independent endocytosis (Bonazzi et al., 2005). PAK1 phosphorylates CtBP1-L on Ser158 (equivalent to Ser147 of CtBP1-S) within a regulatory domain, disrupts dimers, triggers cytoplasmic localization and blocks corepressor functions of CtBP1. RNA interference with two different CtBP1 siRNAs (R1, R2) blocked Ad3 but not Ad5 transduction, and inhibited fluid phase uptake in both infected and non-infected cells (Fig. 10A, B, C). R1 had no effects on Ad3 binding while R2 slightly inhibited Ad3 binding to cells (Fig. 10D). R1 and R2 inhibited Ad3 endocytosis at both low and high moi (Fig. 10E, F). These results were confirmed by expressing the dn D355A mutant of CtBP1-S, which blocked Ad3 but not Ad5 transduction, and fluid phase but not transferrin endocytosis indicating a direct role of CtBP1 in infectious Ad3 endocytosis (Fig. 10G). The expression of the phospho-acceptor defective mutant S147A CtBP1-S but not the phospho-mimetic mutant S147D strongly inhibited Ad3 transduction supporting a role of phosphorylated S147 in the Ad3 infection cascade. Importantly, S147A CtBP1 had no effect on Ad5-eGFP indicating that it interfered with an Ad3 specific process, i.e., infectious Ad3 macropinocytosis. The role of CtBP1 and PAK1 in Ad3 infection was independent of the epithelial cell type, as shown by siRNA knock down in human lung epithelial A549 cells (Fig. 9H). As in HeLa cells, Ad5 but not Ad3 transduction of A549 cells was sensitive to dyn2 siRNA. A knock down of CHC by 78% was, however, not sufficient to inhibit Ad5 or Ad3 infection by more than 20% (not shown).

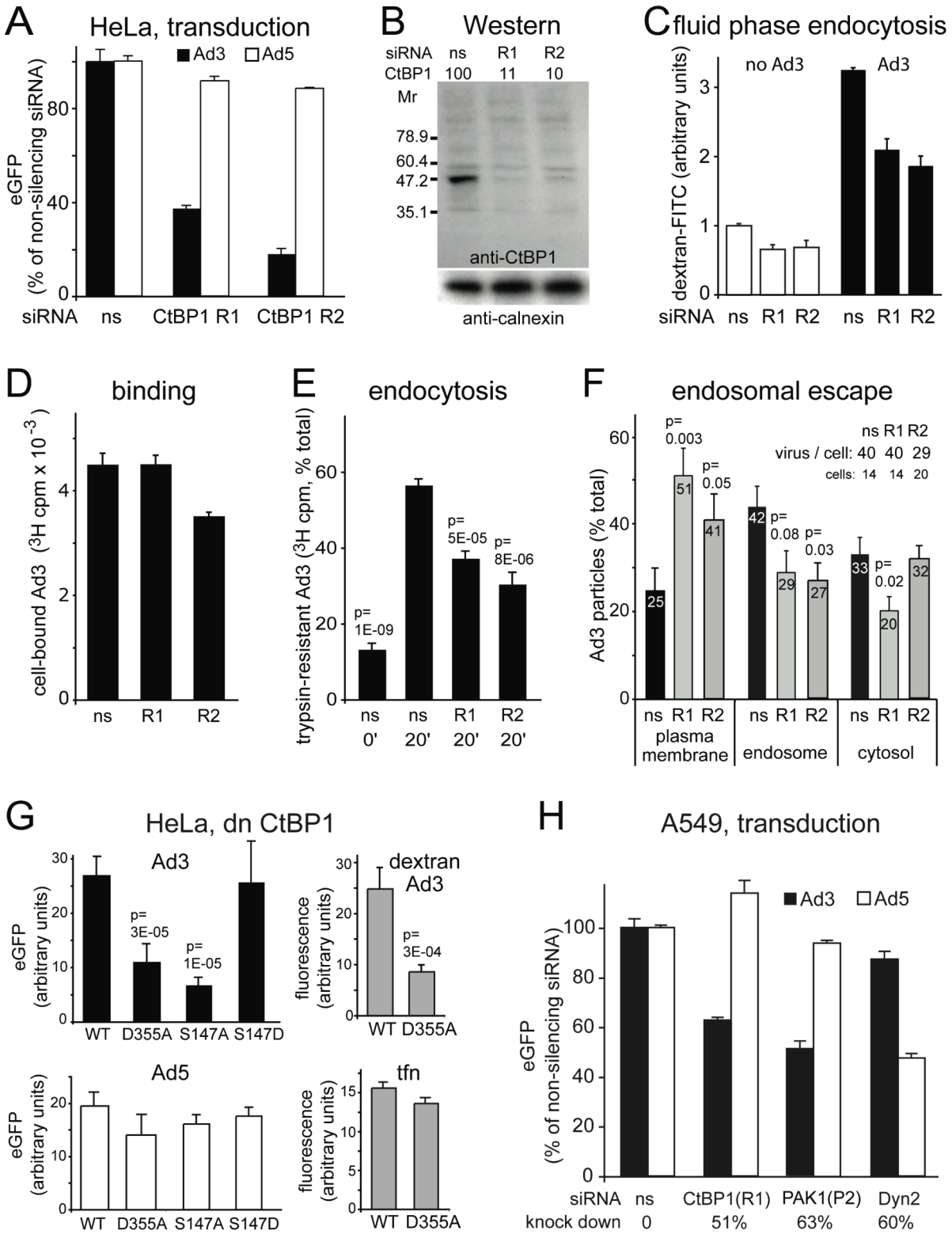

Fig. 10: CtBP1 is required for endosomal uptake and infection of Ad3 but not Ad5.

A: HeLa-ATCC cells transfected with CtBP1 siRNA R1 or R2 were infected with Ad3-eGFP or Ad5-eGFP, and analyzed for GFP expression by flow cytometry. B: Western blot analysis of CtBP1 knock down by siRNA R1 and R2, or ns siRNA, and normalization against calnexin. C: Fluid phase endocytosis of HeLa cells transfected with R1, R2 or ns siRNA. Cells were infected with Ad3 or not infected by cold binding and warming for 10 min in the presence of dextran-FITC, fixed and analyzed for dextran uptake by flow cytometry (10000 cells in triplicates). D,E,F: Binding and endocytosis of (3H)-labeled Ad3 was determined by scintillation counting (10^6 cells, 0.75 µg Ad3), and endosomal

escape of Ad3 by EM in cells transfected with anti-CtBP1 R1, R2 or nonsilencing siRNA, respectively. G: HeLa cells were transfected with myc-tagged CtBP1-S wild type, CtBP1-S D355A mutant, the S147A or S147D mutants for 30 h, infected with Ad3-eGFP or Ad5-eGFP at moi 5 for 16 h, fixed, stained with an anti-myc antibody and analyzed for eGFP fluorescence by confocal laser scanning microscopy and NIH image J analysis of merged set of complete optical sections. CtBP1-S wild type or D355A transfected cells were pulsed with dextran-TR (0.5 mg/ml) or transferrin-Alexa647 (10 µg/ml) for 30 min, fixed and analyzed for dextran and transferrin uptake by confocal microscopy, respectively (grey panels). H: Human lung epithelial A549 cells were transfected with siRNA against CtBP1 (R1), PAK1 (P2), dynamin2, or nonsilencing (ns), transduced with Ad3-eGFP or Ad5-eGFP (moi 2.5), and analyzed for GFP expression by flow cytometry. The levels of knock down were determined by Western blotting using calnexin as a reference.

In noninfected HeLa cells the majority of endogenous CtBP1 was localized to the nucleoplasm (Fig. 11A). In Ad3 infected cells, however, a significant amount of CtBP1 was localized diffusely to the cytoplasm 10 min pi. Most noticeably, CtBP1 was present on the periphery of Ad3-bearing macropinosomes (Fig. 11A, zoomed-in views). The Ad3 induced dextran-positive macropinosomes contained CD46 and alpha v integrin receptors supporting the notion that these vesicles are important for Ad3 infection (Fig. 11B). Noninfected cells contained no detectable pinosomes positive for CD46 or integrin. Together, these data support a role of the membrane organizer CtBP1 in the fission or stabilization of Ad3, CD46, and integrin-containing macropinosomal invaginations and vesicles.

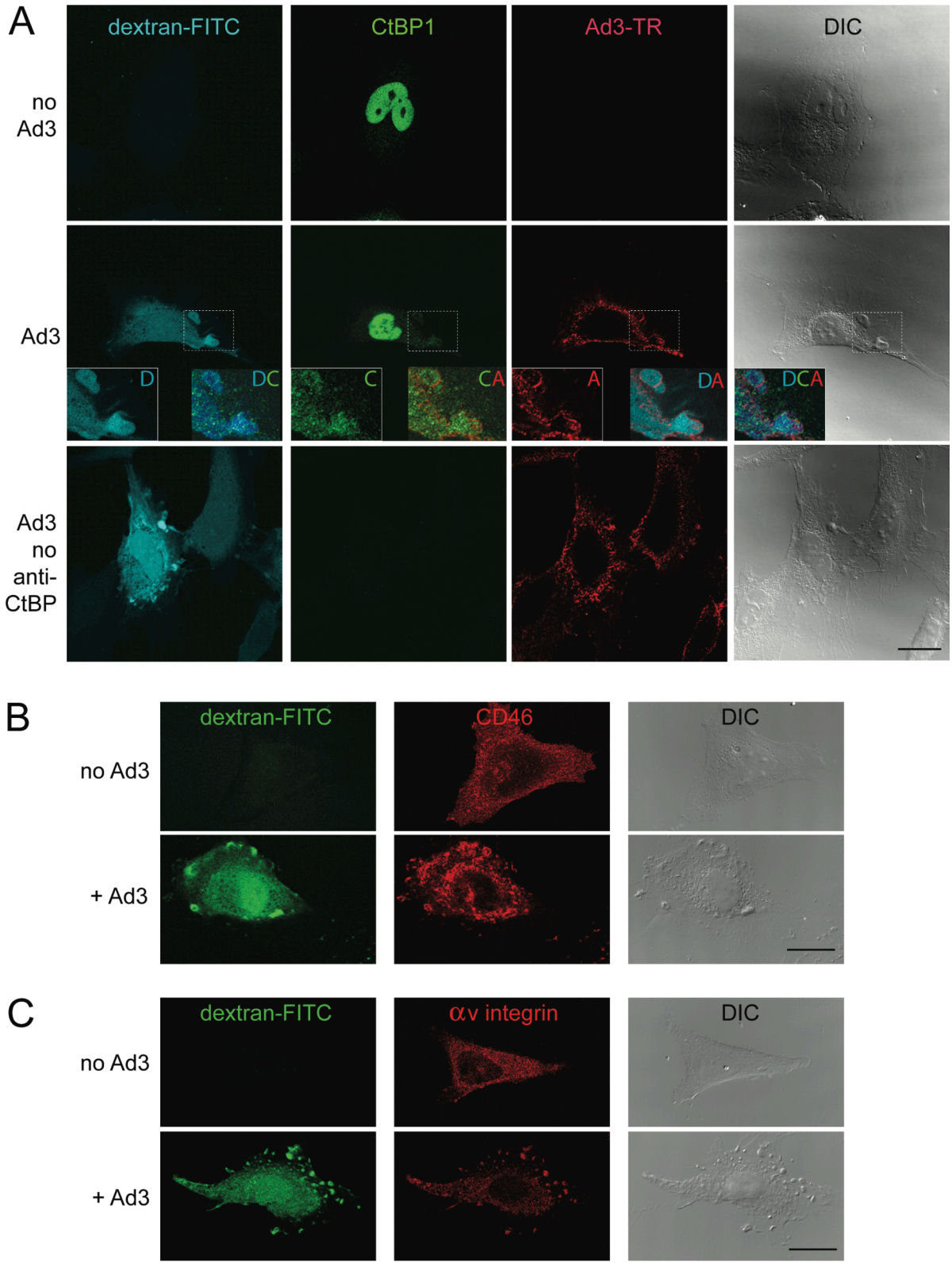

Fig. 11: Ad3 induced macropinosomes contain CtBP1, CD46 and alpha v beta 5 integrins.

Ad3-TR ($2x10^4$ viral particles/cell, moi 50) infected or not infected HeLa-ATCC cells were pulsed with 0.5 mg/ml dextran-FITC at 37°C for 10 min, washed extensively, fixed, immunostained against CtBP1 (0.5 µg/ml), CD46 (mab E4.3, 1 µg/ml), and alpha v beta 5 integrins (mab P1F6, 1 µg/ml) and analyzed by confocal microscopy (A, B, C). The enlarged boxes in panel A show dextran filled macropinosomes (D) containing both CtBP1 (C) and Ad3-TR (A). Control stainings in the absence of

primary anti-CtBP1 antibody are shown in panel A together with the differential interference contrast images (DIC). Bars = 20 µm.

Ad3 infection of hematopoietic cells requires CtBP1 but not clathrin

Human adenoviruses targeting the CD46 receptor have a tropism for hematopoietic cells, unlike the CAR-binding adenoviruses. We found that Ad3 induced a robust transient stimulation of dextran uptake in hematopoietic K562 cells peaking 10 min pi (Fig. 12A). Ad2 in contrast showed a small but significant 2-fold stimulation of dextran uptake, e.g., reflecting the low levels of CAR in these cells (not shown). The Ad3-induced dextran stimulation was dose-dependent, and saturated at approximately 5 µg/ml virus (about 1000 viral particles bound per cell, Fig. 12B). Ad3 transduction of K562 cells was inhibited by CtBP1 siRNAs but not CHC siRNA measured at 16 h pi (Fig. 12C). In contrast, Ad5 transduction was independent of CtBP1 but sensitive to clathrin siRNA, which blocked transferrin receptor uptake (Fig. 12C, D, E). Further, Ad3 transduction of K562 cells was not affected by the dynamin inhibitor dynasore (Macia et al., 2006) up to 200 µM, whereas Ad5-eGFP expression and transferrin uptake were inhibited in a dose-dependent manner (Fig. 12F, G). These results underscore a selective requirement of clathrin for macropinocytosis in certain epithelial but not hematopoietic cells, and a cell type-independent requirement of CtBP1 for infectious macropinocytosis of Ad3.

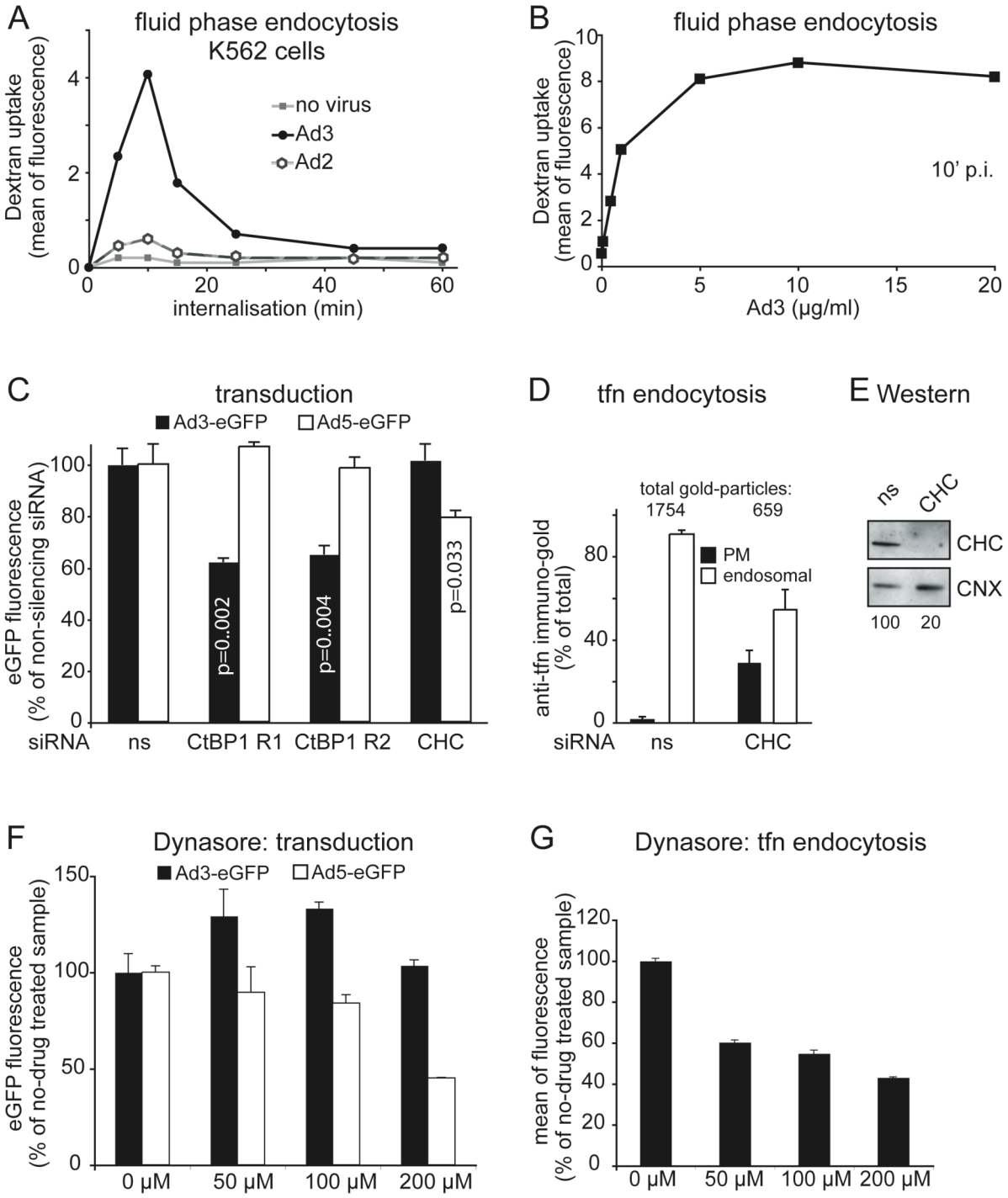

Fig. 12: Infectious Ad3 entry into hematopoietic cells involves fluid phase endocytosis and CtBP1.

A: Hematopoietic K562 cells were infected with Ad3, Ad2 (5 µg/ml virus, equivalent to 2000 viral particles bound per cell) or no virus for different times, pulsed with dextran-FITC (1 mg/ml) for 5 min, and subjected to flow cytometry. B: Dose-dependence of fluid phase uptake stimulation by Ad3 with indicated amounts of Ad3 in the cold, followed by warming for 5 min, and dextran-FITC pulse for 5 min. C: Cells were double transfected with CtBP1 siRNA R1, R2, nonsilencing siRNA (ns) or CHC (CHC) siRNA (20 pmoles/ml) for 48 h, infected with Ad3-eGFP (1 µg/ml) or Ad5-eGFP (2 µg/ml) for 16 h, and analyzed for eGFP expression by flow cytometry. D: Transferrin endocytosis by immuno-EM in CHC or control siRNA treated cells. E: Western blot analyses of CHC siRNA treated cells (1 X 10^5 cells per lane, normalization against calnexin, CNX). F,G: Dynasore block of Ad3-eGFP (1 µg/ml) or Ad5-eGFP (2 µg/ml) infection for 12 h, and transferrin-Alexa488 (10 µg/ml) uptake for 30 min, analyzed by flow cytometry.

Discussion

Endocytic targeting of human adenoviruses depends on the viral fiber protein, and its receptors CAR, and CD46, as well as integrin coreceptors, typically alpha v beta 5 integrins (Berk, 2007; Stewart and Nemerow, 2007). The data here reveal that macropinocytosis is an infectious entry route for the species B human Ad3 in epithelial and hematopoietic cells (Fig. 13). The induction of macropinocytosis is, however, not unique to Ad3, and also occurs with Ad2 or Ad5 (Meier et al., 2002). Unlike Ad3, Ad2/5 binds CAR and primarily internalizes by dynamin-dependent endocytosis. Ad2/5 infection does not depend on the macropinocytosis regulators CtBP1 and PAK1, unlike Ad3 which utilizes macropinocytosis for infection. Both Ad2/5 and Ad3-induced macropinosomes were CtBP1-positive, and they released their fluid contents (data not shown, Meier et al., 2002). In addition, macropinocytic stimulation by Ad2/5 and Ad3 involved alpha v integrins, and Rac1. This implies a common mechanism of adenoviral macropinocytosis, which might have been conserved throughout evolution, considering that the hexon sequences of Ad3 and Ad2/5 are as far away from each other, as the human Ad hexons from mouse, bovine or equine hexon sequences (Davison et al., 2003).

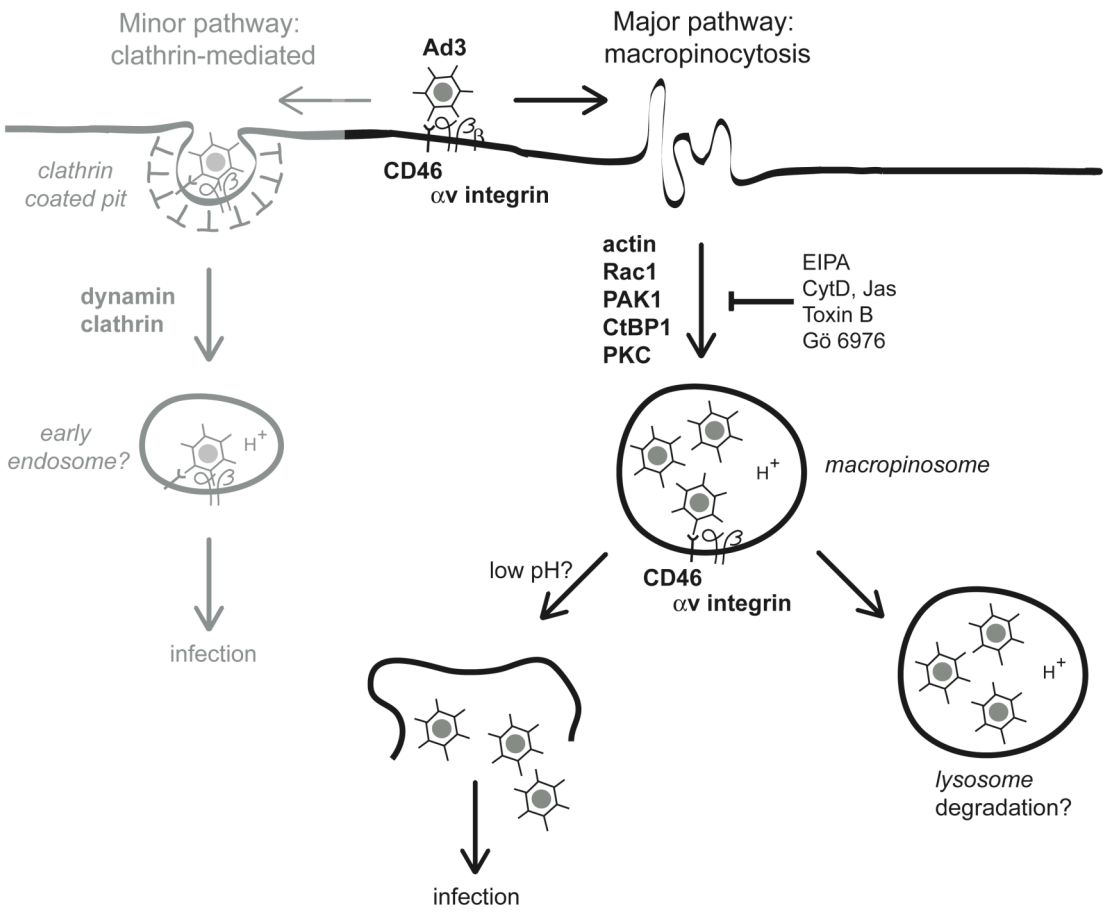

Fig. 13: Macropinocytosis is a major infectious uptake pathway of Ad3 in epithelial cells.

Ad3 binds the membrane cofactor CD46, and is endocytozed into macropinocytic vesicles depending on alpha v beta 3 or beta 5 integrin coreceptors, F-actin, the Rac1 GTPase which activates PAK1 (p21 activated kinase 1), and CtBP1 (C-terminal binding protein 1), a target of PAK1. Protein kinase C (PKC) and the sodium-proton exchanger 1 (sensitive to EIPA) are also required for the formation of Ad3 carrying macropinosomes. Ad3 bearing macropinosomes contain CD46 and alpha v beta 5 integrins. Low pH and additional triggers probably lead to virus release from macropinosomes, and noninfectious virions are degraded in late endosomes and lysosomes. Besides the major macropinocytic pathway there is a minor clathrin and dynamin-dependent pathway, which is cell type-dependent and not found in K562 hematopoietic cells, for example. This pathway remains to be characterized.

The stimulation of infectious macropinocytosis by Ad3 required both the CD46 receptor and alpha v integrin coreceptors. Ligation of both CD46 and alpha v integrins by antibodies induced fluid phase uptake suggesting that CD46 and integrins can provide upstream signals for macropinocytosis. In fact, integrins have been implicated in macropinocytosis and phagocytosis of dead cells (Wu et al., 2006). CD46 on the other hand is a well known pathogen receptor. It binds vaccine strains of measles virus, human herpes virus 6, the species B human adenoviruses

and bovine viral diarrhea virus binding to CD46 (Cattaneo, 2004). Binding to CD46 may be attractive for pathogens since CD46 impairs activation and effector function of T-cells, and T-cell response to NK cells (Oliaro et al., 2006). CD46 signalling is also intimately linked to endocytosis. Depending on the degree of ligand crosslinking, CD46 engages in constitutive clathrin-mediated endocytosis, membrane ruffling, or macropinocytosis (Crimeen-Irwin et al., 2003). CD46 was however not sufficient for Ad3 endocytosis. Infectious Ad3 uptake also required alpha v integrins. It remains to be determined if integrins alone or in conjunction with other cell adhesion molecules signal macropinocytic uptake in other viral and bacterial infections.

Macropinocytosis has strict requirements for actin, Rac1, protein kinase C, and the sodium/proton exchanger extruding protons from the cytosol. Proton efflux from the cell through the sodium/proton exchanger is an evolutionarily conserved mechanism to regulate cytoskeleton dynamics, cell migration, growth factor induced proliferation, and the formation of the phagocytic/macropinocytic cup (Baumgartner et al., 2004; Denker and Barber, 2002). It is possible that actin polymerisation controlled by Rho GTPases provides the driving force for membrane protrusions, and that the actin-anchored sodium-proton exchanger (and perhaps other ion exchangers) contribute to localized volume changes near the plasma membrane. In this scenarium, the sodium-proton exchanger would be upstream of Rac1-PAK1-CtBP1 signalling by Ad3. PAK1 activation then triggers CtBP1 translocation to the cytoplasm which coincides with transcriptional derepression (Barnes et al., 2003). The observations that Ad3 but not Ad5 infection was inhibited by dn S147A CtBP3 suggests that Ser147 phosphorylation is important for infectious entry of Ad3. In addition, both Ad3 and Ad2/5 activated PAK1, and CtBP1/3 was found on Ad3 or Ad2/5 induced macropinosomes (the Ad2/5 data are not shown). This suggests that the recruitment of CtBP1 to macropinosomal membranes may contribute to derepression of host genes during Ad2/5 and Ad3 entry.

CtBP family members are important regulators of innate immunity. They bind the immediate early viral transactivator E1A of all primate and human adenoviruses (Berk, 2005; Chinnadurai, 2003), and regulate the expression of the interferon

regulatory factor 3 (IRF3) gene (Johansson et al., 2005). Additional CtBP targets include transcriptional coactivators, such as histone acetyl transferases, and the E1A associated protein p300 (Chinnadurai, 2002; Senyuk et al., 2005). CD46 and CAR binding adenoviruses induce a strong TLR9-dependent immune response involving NFkB and various cytokines (Iacobelli-Martinez and Nemerow, 2007). Interestingly, incoming adenoviruses activate protein kinase A which enhances p300/CBP activity (Mayr and Montminy, 2001; Suomalainen et al., 2001). It is conceivable that PAK1 mediated activation of CtBP1 synergizes with activated TLR9, PKA and PAK1 to stimulate histone acetylation. This could account for the observation that adenovirus infected epithelial cells and fibroblasts upregulate a number of CtBP1 repressed genes, such as the CCAAT/enhancer binding protein beta, which binds to p300/CBP and CtBP1, and thus could contribute to the establishment of an anti-viral state (Granberg et al., 2006; Schuierer et al., 2001; Zhao et al., 2003). We suggest that CtBP1-dependent macropinocytosis is a defense reaction against pathogens that cause transcriptional derepression of innate immunity genes. The ubiquitous nature and high capacity of macropinocytosis in both professional and nonprofessional antigen presenting cells suggest that it might serve pathogens as an entry gate into different cell types, including polarized epithelial cells (Bruewer et al., 2005). The results here may have implications for understanding how endocytic pathogen uptake is connected to the innate immune response in both pathological and therapeutic settings, including gene delivery and vaccination.

Materials & Methods

Cells and viruses

Human melanoma M21 litter (negative for surface-expressed αv integrins) and M21L cells (positive for cell surface αv integrins) were from Dr. D. Cheresh (Scripps Research Institute, La Jolla, CA, USA). Cells were grown in DME (GIBCO-BRL) containing 10% FCS (GIBCO-BRL) at low passage number as described (Meier et al., 2002). K562 chronic myelogenous leukemia cells were grown as described (Meier et al., 2005). Ad3 and Ad2ts1 were grown, labeled and isolated as described (Meier et al., 2002; Suomalainen et al., 2001). We estimated that 1000 Ad3 particles were equivalent to 2 infectious particles bound per cell (moi 2), based on a particle to infectious particle ratio of 20, and the observation that 4% of radiolabeled Ad3 bound to cultured human epithelial cells in any given experiment.

cDNAs, proteins and chemicals

cDNAs encoding CtBP1-S were obtained from Dr. A. Colanzi (Department of Cell Biology and Oncology, S. Maria Imbaro, Italy). pCMV-myc CtBP1-S wt was generated by ligation of the PCR amplified CtBP1-S wt (digested with Sal I and Not I, respectively) into the pCMV backbone vector (Stratagene). Myc-CtBP1-S D355A was generated with the QuikChangeR site-directed mutagenesis kit (Stratagene) with the primers 5'-CTGGGCCAGCATGGCCCCTGCTGTGGTG-3' and 5'-CACCACAGCAGGGGCCATGCTGGCCCAG-3' (Bonazzi et al., 2005). The obtained cDNA was verified by sequencing. PAK1 wt and inhibitory domain expression vectors were from J. Chernoff (Fox Chase Cancer Center, Philadelphia, PA). K44A-dyn2 and dyn2 wt expression plasmid were from Dr. C. Lamaze (Pasteur Institute, Paris, France). eGFP-eps15deltaEH2,3 and eGFP-eps15DIIIdelta2 cDNAs were from Dr. A. Benmerah (Institut National de la Santé et de la Recherche Médicale E9925, Paris, France, Benmerah et al., 1999), amphiphysin expression plasmids were from Dr. P. De Camilli (Yale University, New Haven, USA). Expression plasmids encoding Rac1 wt or dominant-negative T17N and Cdc42 wt

or dominant-negative T17N enhanced by eCFP were from Dr. A. Hall (University College, London, UK). The Arf6 constructs were obtained by Dr. J. Donaldson (NIH, Bethesda, MD, USA). The PKC inhibitors Gö 6976 (1 µM) and Gö 6983 (1 µM) were purchased from Calbiochem (Juro Supply), the Na+/H+ exchanger inhibitor EIPA (100 µM) was from Alexis Corporation, Cytochalasin D (5 µM) and Jasplakinolide (500 nM) from Calbiochem. Cholesterol depletion by methyl-beta-cyclodextrin (50 mM) was performed as published earlier (Imelli et al., 2004). Dynasore was kindly synthesized by Dr. Jay Siegel (Institute of Organic Chemistry, University of Zurich, Switzerland). Cells were pretreated with inhibitors in RPMI-BSA at 37° C for 30 min, infected for 60 min in presence of drugs followed by washing in medium without drug and further incubations as indicated. Antibody against CtBP1 were from BD Transduction laboratories, PAK1 antibody (C-19) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibody against T423 phophorylated PAK1 (Cell Signaling Technology, Danvers, MA, USA). The anti-calnexin antibody was a gift of Ari Helenius (ETH Zürich, Switzerland). Toxin B (0.5 mg/ml) was from Drs. F. Hofmann and K. Aktories (University of Freiburg, Freiburg, Germany). Ad3 soluble fiber knob used at a final concentration of 5 µg/ml was from P. Fender (Grenoble, France).

Transduction, fluorescence microscopy and immunofluorescence

eGFP transductions and analyses by flow cytometry or single cell confocal microscopy were carried out as described (Meier et al., 2005). Shortly, cells were transfected with different DNA constructs 30 h prior to experiment (or if different, as described) using Fugene 6 (Roche, according to manufacturer's instruction), infected with Ad3-eGFP or Ad5-eGFP at 37° C for 60 min, washed and incubated at 37° C for 15 h. Cells were fixed and mounted with DAKO. For dextran and transferrin uptake, cells were synchronized with 5 µg/ml of Ad3 in the cold, washed warm and pulsed with a mixture of 0.5 mg/ml dextran-TR and 20 µg/ml of transferrin-Alexa647 in RPMI-BSA at 37° C for 30 min (waterbath), followed by a 5 min chase, fixed and mounted with DAKO. Confocal laser scanning microscopy was

performed on a Leica-DM SP2 RXA2-TCS-AOBS microscope (Leica Microsystems, Wetzlar, Germany) equipped with an Ar-ArKr laser, a He-Ne 543-594 laser, a He-Ne 633 laser, a diode laser at 405 nm, and a 63 x oil immersion objective (N.A. 1.4 PL APO). The pinhole value was 1.0, airy 1, yielding optical sections of ~0.48 µm with a voxel of 0.233 by 0.233 by 0.48 µm. The zoom factor was 2. Image processing was performed with Leica and Photoshop software (Adobe), and fluorescence intensities determined using NIH Image J (http://rsb.info.nih.gov/ij/) on cell total projections. For CtBP1 colocalization with dextran-positive endosomes cells were cold synchronized with Ad3-TR (2 µg/ml) for 60 min on ice, washed with warm RPMI-BSA and pulsed with 0.5 mg/ml of dextran-FITC at 37° C for 10 min. Cells were washed extensively with RPMI-BSA and PBS, fixed and analyzed by immunofluorescence using a CtBP1 mouse monoclonal antibody (BD Transduction laboratories) and a secondary Alexa647-conjugated goat anti-mouse antibody.

siRNA transfections

K562 cells were transfected with siRNA directed against clathrin heavy chain (AACCUGCGGUCUGGAGUCAAC; Qiagen, (Meier et al., 2005)) and against CtBP1 (CCGUCAAGCAGAUGAGACAUU; GGAUAGAGACCACGCCAGUUU (Dharmacon) (Bonazzi et al., 2005)) using Nucleofector I (Amaxa; program T-03) according to the manufacturer's instructions, using non-silencing siRNA sequences (Qiagen, or Dharmacon) as controls. Transfections were done at day 0 and day 2, cell lysates for Western blotting and experiments were collected at day 4. HeLa cells were transfected with siRNA directed against clathrin heavy chain, CtBP1/CtBP3 or PAK1 (validated siRNA Cat. SI00605703 and SI00605696; Qiagen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfections were done twice at day 0 and day 2, cell lysates for Western blotting and experiments collected at day 4. A549 cells were transfected with siRNA directed against clathrin heavy chain, CtBP1/CtBP3, PAK1 or dynamin2 (GACAUGAUCCUGCAGUUCA, Qiagen) using Lipofectamine 2000.

Preparation of BSA-gold, Transferrin uptake

15 nm collodial gold was prepared by citrate reduction of HAuCl4 (Horisberger and Rosset, 1977). To 20 ml of collodial gold solution (pH adjusted to 5.9) 50 µl of 10 mg/ml BSA (Sigma, fatty acid free) solution was added (De Roe et al., 1987). To stabilize the BSA-gold complex, 1ml of 1% PEG 20000 (Roth, Switzerland) were added, the sample centrifuged at 28'000 g for 60 min, and the pellet dissolved in 2 ml gold-buffer (sterile filtered PBS containing 0.2% PEG-20000) and stored at 4° C. BSA-gold internalization was performed after cold binding of Ad3 or Ad2-ts1 using a 1:1 dilution of BSA-gold with RPMI-BSA (approximately 0.1 mg/ml of BSA) at 37° C for 10 min. Transferrin endocytosis was determined by binding a mouse monoclonal anti-transferrin receptor antibody (Thomas Ebel, Karolinska Institute, Huddinge, Sweden) in the cold for 30 min, followed by a secondary anti-mouse antibody coupled to 10 nm gold particles as described (Meier et al., 2005). Antibody-receptor complexes were internalized at 37° C for 30 min, and quantified for plasma membrane or endosomal localization of gold particles in a total of 5 different cells for each condition.

Endocytosis and Ad-eGFP transductions

Cells were incubated with (3H)-thymidine-labeled Ad3 (1 µg/ml; 3 X 109 particles on a 35 mm dish) in the cold for 60 min in RPMI-0.2%BSA, washed with cold RPMI-BSA and warmed with RPMI-BSA for the indicated time points. Cells were washed twice with cold RPMI-BSA and cold PBS. To remove the extracellular virus particles from the cells, cells were incubated with cold 2% trypsin-EDTA (GIBCO) for 60 min in the cold, PBS-2%FCS was added and the cells centrifuged at 500 x g. The wash step was repeated twice. Cell lysates were prepared in hot SDS (0.4%), sheared in a 20G clinical syringe, and radioactivity was determined by fluid scintillation counting (Ready Safe; Beckman Coulter) with a Beckman Coulter Scintillation System LS 3801. Counts of control cells without trypsin were used as 100% control. For transduction experiments cells were washed with warm RPMI-BSA and incubated with 1 µg/ml of virus for 60 min, washed several times with RPMI-BSA

and incubated in a water bath for 4 h (A549 cells), 5 h (HeLa cells), 8 h (M21 and M21L cells) and 16 h (K562 cells). The cells were washed with cold PBS and treated with 2% trypsin in the cold, followed by 2% PBS-FCS and analysis by flow cytometry (Beckman FC500 cytometer). At least 10000 viable cells were counted per sample. For drug experiments cells were pretreated with inhibitors in RPMI-BSA at 37° C for 30 min, followed by warm infection for 60 min in presence of drugs followed by washing in mediumm without drug and further incubation.

Dextran uptake

Cells were preincubated with 5 µg/ml Ad3 in the cold, washed with warm RPMI-BSA, and warmed in RPMI-BSA containing 0.5 mg/ml dextran-FITC (lysine-fixable 10 kDa, Molecular Probes) at 37°C for 10 min, as described earlier (Meier et al., 2002). Dextran uptake was stopped by washing cells with cold RPMI-BSA and PBS (3 repeats). Surface-bound dextran was removed by acid treatment in cold 0.1 M sodium acetate pH 5.5, 0.05 M NaCl for 10 min. For FACS analysis, cells were detached with 2% trypsin in PBS (GIBCO-BRL) on ice for 25 min, transferred into 6 ml polypropylene tubes (no 2063; Falcon, Becton Dickinson) containing 2 ml of 7% FCS/PBS, pelleted at 290 x g and resuspended in 2 % FCS/PBS. At least 10000 viable cells were counted per sample in a flow cytometer (Beckman FC500 cytometer).

Nuclear targeting assays

HeLa cells were transfected with different plasmids encoding GFP-tagged proteins of interest for 24 h, followed by cold synchronized Ad3-texas red infection for 150 min, and analysis of nuclear targeting as described (Nakano and Greber, 2000).

Transmission electron microscopy

After cold binding of Ad3 or Ad2 ts1 (50 µg/ml, moi 5000) for 60 min, washing and internalization as appropriate, cells were fixed in 2% formaldehyde-1.5 %

glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 (CaCo) overnight, and washed several times in CaCo, followed by postfixation in 1% OsO4 (Electron Microscopa Sciences) and 1.5% potassium ferricyanide (FeK3N6) in double distilled water at 4° C for 60 min (modified according to the medhod of Simionescu and Siminonescu). Specimens were rinsed in 0.1 M sodium cacodylate, contrasted with 1% tannic acid in 0.05 M sodium cacodylate at room temperature for 45 min, washed in 1% sodium sulfate, rinsed in H2O, stained in 2% uranylacetate in H2O over night, and embedded in Epon as described previously (Meier et al., 2005). Virus particles were quantified at 50000 x magnification in ultrathin sections at the plasma membrane, endosomes and cytosol, and viewed in a transmission electron microscope (Zeiss EM 902A) at an acceleration voltage of 80,000 V. Surface staining of HeLa cells with cationized ferritin (Sigma) was performed after Ad3 cold binding for 60 min and warming for 10 min in RPMI-BSA. After two cold washes with RPMI-BSA cells were incubated with ferritin at 2.5 mg/ml on ice for 10 min.

Western blotting

Cells were grown in 35-mm dishes, washed with phosphate-buffered saline (PBS) and lysed in 500 µl of 2% hot SDS. The lysate was passed through a 20-gauge needle several times and heated to 95°C for 30 s. After centrifugation at 16,000 × g for 10 min, 150 µl of the supernatant was mixed with 50 µl of sample buffer (200 mM Tris/HCl, pH 6.8, 8% SDS, 0.4% bromphenol blue, 40% glycerol, 167 mM dithiothreitol) and heated to 95°C for 10 min. Extracts were separated on 10% SDS-PAGE, transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Zurich, Switzerland), and blocked with 5% dried milk in 50 mM Tris/100 mM sodium chloride/0.1% Tween, pH 7.4 (TNT). After immunological probing (with 3 % milk for the PAK1 blots, with 0.2 % BSA for the CtBP1 blots) HRP-conjugated antibodies were detected with ECL Plus reagents (Amersham Biosciences). Filters were stripped with 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris/HCl, pH 6.7, at 50°C for 30 min, washed extensively with TNT, blocked with 5% dried milk, and reprobed with a control antibody. For PAK1 activation, 5 X

105 HeLa cells were incubated with 0.5 µg/ml of Ad3, Ad5 or ts1, washed, internalized, chilled with cold PBS containing phosphatase and protease inhibitors, scraped off the dish, resuspended in 100 µl of PBS with inhibitors, mixed with SDS sample buffer, heated and analyzed by SDS-PAGE (105 cell equivalents per lane), and ECL as recommended by the manufacturer (Amersham).

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Part II

Ad35 endocytosis

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Keywords: infectious disease / endocytosis / cell defense / transcription / innate immunity

The following have contributed to the figures of the Results section:

Figure 1: Beat Amstutz, Stefan Kälin

Figure 2: Karin Boucke, Beat Amstutz

Figure 3: Michele Gastaldelli, Beat Amstutz

Figure 4: Michele Gastaldelli, Nicola Imelli

Figure 5: Michele Gastaldelli, Nicola Imelli

Figure 6: Michele Gastaldelli, Nicola Imelli

Figure 7: Beat Amstutz

Manuscript in preparation

Abstract

Endocytosis supports cell communication, growth, and pathogen infection. The species B human adenovirus serotype 35 (Ad35) causes urinary tract and kidney infections, as well as lung infection in immunocompromised individuals. Ad35 uses CD46 and integrins for infection of epithelial and hematopoietic cells. Here we show that Ad35 uses both dynamin-independent and dynamin-dependent endocytosis for infectious entry into epithelial cells. It stimulates fluid phase uptake and is found in vesicles containing the fluid phase marker BSA-gold. Infectious Ad35 entry requires p21-activated kinase 1 (PAK1), and the C-terminal adenoviral E1A binding protein 1 (CtBP1), a bifunctional protein involved in membrane traffic and transcriptional repression of cell cycle, cancer and innate immunity related signalling pathways. Together with previous evidence that Ad3, another species B Ad uses macropinocytosis for infectious entry the data underscore that fluid phase uptake is a common pathway for species B Adenovirus infection. We suggest that both CD46 and integrins are required to direct species B adenoviruses to infectious macropinocytosis in epithelial and hematopoietic cells.

Introduction

Animal cells support a variety of endocytic pathways to coordinate signal transduction, cell growth, differentiation, death, and also pathogen infection (Marsh and Helenius, 2006). The first discovered and best characterized endocytic pathway is the clathrin dependent pathway. It funnels ligand receptor complexes into acidic early and late endosomes and lysosomes. It is used by pH-dependent animal viruses, including Semliki Forest virus (Helenius, 1980), vesicular stomatitis virus (Superti, 1987), hantaan virus (Jin, 2002), influenza virus (Matlin, 1982), bovine viral diarhea virus (Lecot, 2005), parvoviruses (Parker, 2001; Bantel-Schaal, 2002), human rhinovirus type 2 (Snyers, 2003), foot and mouth disease virus (Berryman, 2005), human papilloma virus (HPV) types 16 and 58 (Bousarghin, 2003), reovirus (Ehrlich, 2004) and adenovirus type 2 and 5 (Varga, 1991; Meier, 2002).

Other endocytic pathways include the dynamin dependent pathways phagocytosis, interleukin-2 endocytosis and route of intercellular adhesion molecule 1, caveolar and lipid raft-dependent uptake (Gruenberg and van der Goot, 2006; Orth et al., 2006; Parton and Simons, 2007). Only a few dynamin-independent endocytic uptake mechanism are known, e.g. the cdc42-dependent pathway leading to the GPI-anchored protein enriched early endosomal compartments (GEECs; Sabhranjak et al., 2002), or macropinocytosis that engulfs large amounts of extracellular fluid (Schnatwinkel et al., 2004). Macropinocytosis can be induced by growth factors, Ras or Src tyrosine kinase (Patel and Galan, 2006). In viral infections, macropinocytosis is an accessory pathway triggered by HIV (Liu et al., 2002; Marechal et al., 2001), and human adenovirus types 2 and 5 (Ad2/5) in respiratory epithelial cells (Meier et al., 2002). It has remained unclear, however, if macropinocytosis is an infectious entry pathway for pathogens.

Adenoviruses are a diverse family of agents with more than 50 serotypes identified in humans. They infect epithelial cells of the upper and lower respiratory tracts, the urinary and digestive tracts, and a variety of blood cells (Gray et al., 2000; Kim et al., 2003; Uhnoo et al., 1990; Marini et al., 2002).

Adenoviruses are associated with exacerbations of asthmatic conditions and additional clinical syndromes - most notably infantile gastroenteritis with associated morbidity, and local epidemics, particularly in crowded areas with poor hygiene. The species B serotype Ad35 is associated with urinary tract and kidney infections that are often fatal in immunocompromised individuals (Hierholzer, 1992). Importantly it has a low seroprevalence and Ad35-derived vectors are among the most promising candidates for adenoviral gene therapy (Seshidhar Reddy et al., 2003).

For cell entry, Ad35 uses the transmembrane protein CD46 for attachment receptor (Fleischli et al., 2007; Gaggar et al., 2003). CD46 protects autologous cells from complement attack, and links innate and acquired immunity in macrophages and lymphocytes (Liszewski and Atkinson, 1996; Riley-Vargas et al., 2004). Many different viruses bind CD46 for productive infection, e.g. measles virus (Edmonton strain), human herpesvirus 6 or bovine viral diarrhea virus (Cattaeno 2004). Additionally it contains binding sites for complement factors C3b and C4b and serves as a receptor for Strepptococcus pyogenes and pathogenic Neisseria bacteria. CD46 is continuously endocytosed into clathrin-coated pits and upon crosslinking downregulated by macropinocytosis (Crimeen-Irwin et al., 2003).

Here we show that infectious endocytic uptake of Ad35 occurs through macropinocytosis, and dynamin- and clathrin- dependent endocytosis. It is controlled by a transcriptional corepressor, the C-terminal binding protein 1 of E1A (CtBP1). Two mammalian genes of CtBP exist, CtBP1 and CtBP2, each occurring in two splice forms, long and short (CtBP1-L/S and CtBP2-L/S, respectively). They have partly overlapping transcriptional functions in tumorigenesis, apoptosis, development, cell differentiation and viral infection (Berk 2005).

The short version of CtBP1-S (also called brefeldin A-ribosylated substrate, BARS) lacks 11 N-terminal amino acids, and like CtBP1-L is involved in dynamin-independent endocytosis (Bonazzi et al., 2005; Chinnadurai, 2002). In normal cells, CtBP1 heterodimerizes with CtBP2, and shuttles between the nucleus and the cytoplasm depending on posttranslational modifications and binding to PDZ proteins (Barnes et al., 2003; Verger et al., 2006).

Results

Ad35 uses the two outer complement control protein repeats (CCPs 1 and 2) of CD46 for primary attachment and infectious endocytosis (Fleischli et al., 2007; Gaggar et al., 2005). Since a deletion of the cytoplasmic tail of CD46 caused no apparent change in infection of nonpolarized cultured cells (Sakurai et al., 2005), it is likely that Ad35 needs secondary receptors for infectious entry. Adenovirus type 2/5 were shown to expose an RGD-motif in the penton base and bind to alpha v integrins. Ad35 contains an RGD-motif in the penton base like Ad2/5 or Ad3. Thus, we tested if alpha v integrin were involved in infectious Ad35 endocytosis. Ad35-eGFP transduction of alpha v integrin deficient, CD46 positive M21L melanoma cells was severely reduced compared to alpha v integrin positive M21 cells, although binding was not strongly affected (Fig. 1A, B). Similar results were obtained with Ad5-eGFP and Ad3-eGFP (first part of this thesis). Interestingly, Ad35 binding is slightly higher on alpha v expressing M21 cells, in comparison to Ad3 that bound more efficiently to M21L cells (first part of this thesis, Figure 1A). Despite the slight difference in binding, Ad35 could not be released by trypsin treatment from HeLa-ATCC cells unlike Ad3 (unpublished data by S.Kälin, F. DiGennaro and B. Amstutz, respectively; Segerman et al., 2003).

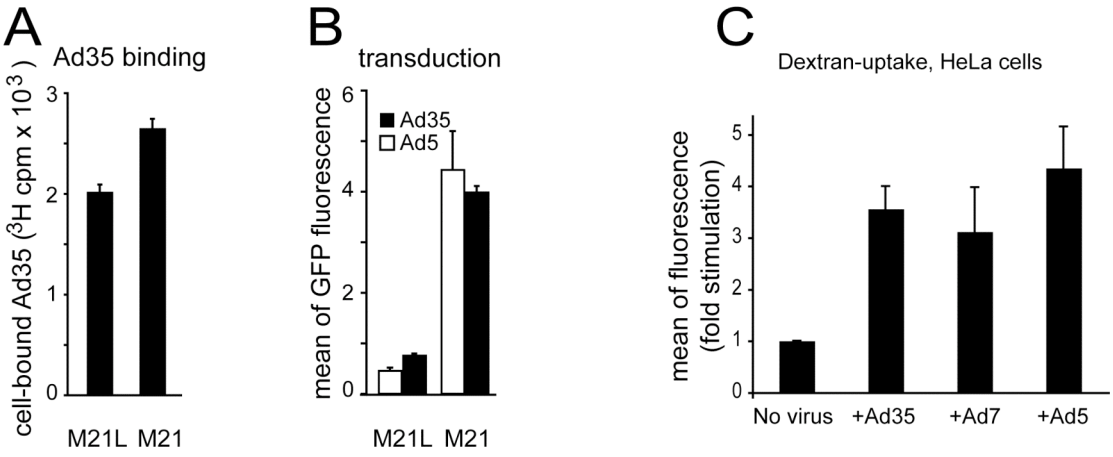
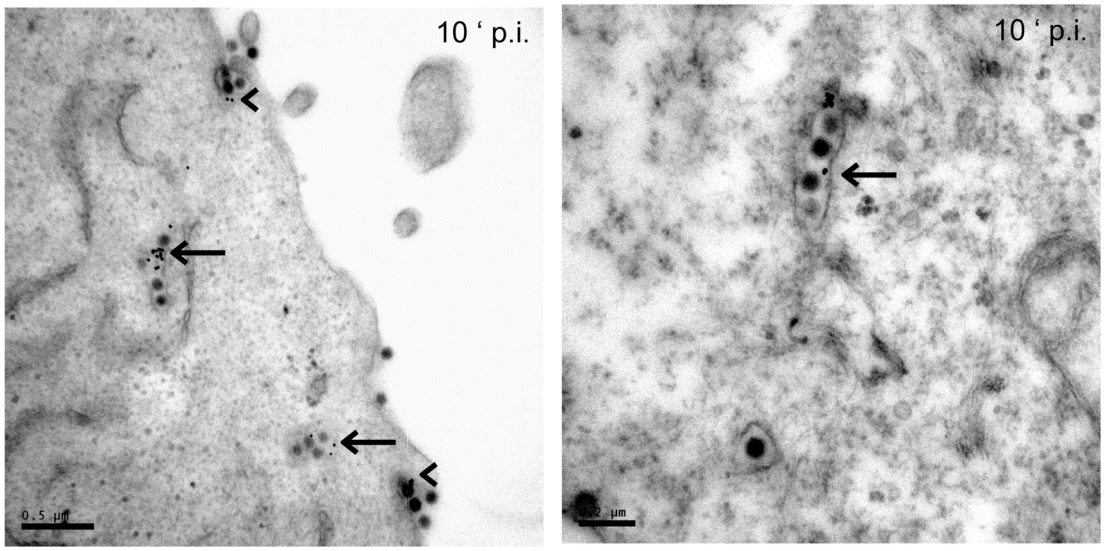


Figure 1. Ad35 infection requires alphav integrins and coincides with fluid phase uptake stimulation. A: Human melanoma M21 or M21L cells positive or negative for alpha v integrins, respectively, were incubated with (3H)-labeled Ad35 in the cold, and analyzed for cell-associated radioactivity. B: M21 or M21L cells were transduced with Ad35-eGFP or Ad5-eGFP (moi 5) and analyzed by flow cytometry. C: Dextran uptake in HeLa-ATCC cells is stimulated by Species B Ad7 and Ad35 and Species C Ad5, respectively. HeLa-ATCC cells with cold bound Ad35, Ad7, Ad5 or no virus (5 µg/ml virus, equivalent to 2000 viral particles bound per cell for 60 min in cold medium followed by washing) were warmed for 5 min and

pulsed with dextran-FITC (0.5 mg/ml) for 5 min, washed in isotonic sodium acetate pH 5.5, and subjected to flow cytometry analysis.

Since adenoviruses of both species C Ad2 and species B Ad3 stimulate fluid phase uptake in epithelial cells we infected HeLa-ATCC cells with Ad5, Ad7 and Ad35, two further Species B Ads, and measured the uptake of dextran-FITC by FACS analysis. As expected, Ad5 stimulated fluid phase uptake (Fig. 1C). Both Ad7 and Ad35 stimulated fluid phase uptake unlike earlier published data (Defer et al., 1990). Pulsing Ad35 infected HeLa-ATCC cells with saturating BSA-gold for 10 min revealed high amounts of viral particles within endosomes colocalizing with gold-particles (data not shown) like it was found with Ad3 but not Ad2 ts1, a virus deficient in fluid phase uptake stimulation (first part of thesis, Meier et al., 2002). Additionally a few viruses were found within coated pits (< 3% of total virus, data not shown).

A



B

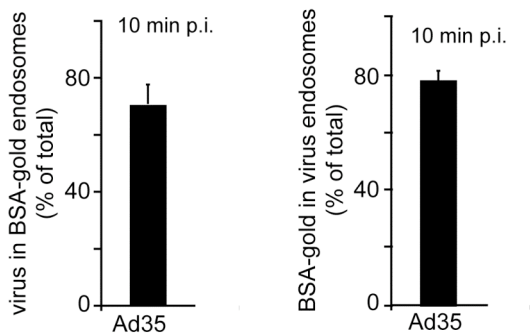


Figure 2: Ad35 is found in endosomes rich in fluid phase marker BSA-gold.

A, B: HeLa-ATCC cells were incubated with Ad35 in the cold, washed, pulsed with BSA-gold for 10 min, fixed for ultra thin section EM analyses, and quantified for viral particles in gold positive endosomes (arrows), and gold particles were counted in endosomes carrying Ad3. Viruses in plasma membrane invaginations are pointed out by arrow head.

To test to which extent clathrin- and dynamin-dependent endocytic mechanisms were involved in Ad35 endocytosis we made use of different approaches. First, overexpression of dominant-negative dynamin2 K44A (dn). Transferrin uptake and infection of Ad5-eGFP was strongly reduced in cells overexpressing dynamin dn, whereas Ad35-eGFP infection was not reduced significantly in HeLa-ATCC using a single cell confocal assay (Fig. 3A). Similar results were obtained using Ad3-eGFP (first part of thesis). However, similar to Ad3-eGFP infection, Ad35-eGFP and Ad5-eGFP infection in HeLa-K cells was sensitive to dynamin K44A as measured by a FACS-approach (figure 3B, C). To further investigate the requirement for dynamin in Ad35-eGFP infection in the original HeLa-ATCC cells, siRNA interference with siRNA against dynamin was performed. Both the Ad35-eGFP and Ad5-eGFP infection in these cells was reduced to 50% (figure 4A). Quantification of electron micrographs showed that 75% of Ad35 was at the plasma membrane in dyn2 siRNA treated cells, showing that the internalization of Ad35 was strongly reduced (fig. 4B).

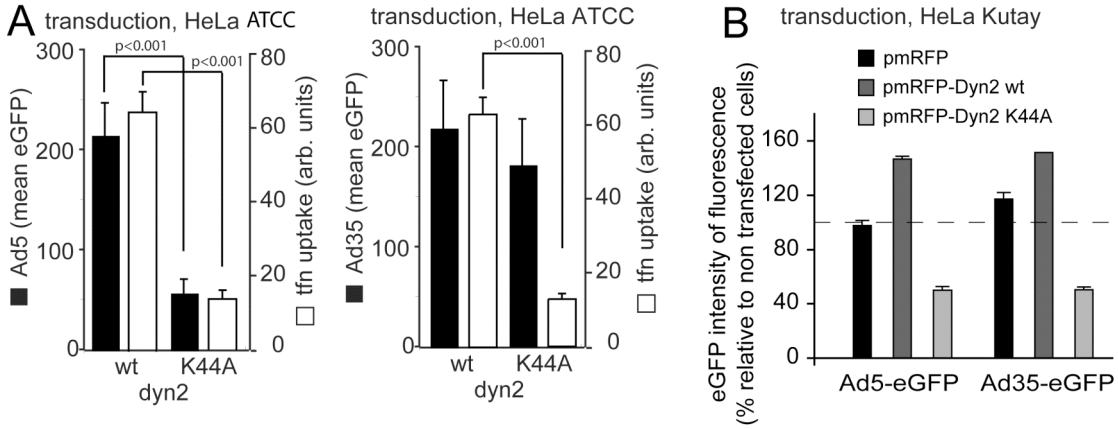


Figure 3. Differential requirement of dynamin for Ad35 and Ad5 infections, respectively, in HeLa-ATCC and HeLa-K cells. A: Ad5-eGFP and Ad35-eGFP transduction (12 h) and transferrin-647 internalization (10 µg/ml transferrin in the last 30 min of infection) in HeLa-ATCC cells transfected with wt dynamin2 (wt dyn2), or K44A dyn2 for 40 h, analyzed by confocal microscopy in single cell assays (moi 5). B: Ad5-eGFP and Ad35-eGFP transduction (6h) in HeLa-K transfected with wt dynamin2 (dyn2) or K44A dyn2 for 48 h, and analyzed by FACS-analysis. 10000 cells per each sample were analyzed and quantification of FACS analysis is shown.

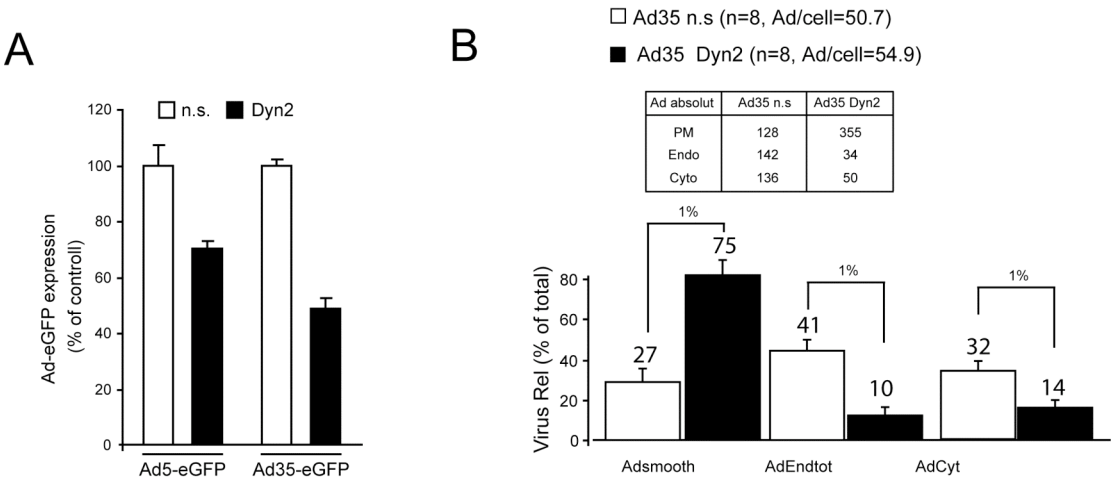


Figure 4. siRNA knockdown of dynamin2 reduces both Ad5 and Ad35 infection in HeLa-ATCC cells. A: Ad5-eGFP and Ad35-eGFP transduction (6 h) in HeLa-ATCC treated with either non-silencing (n.s.) or dynamin2 (dyn2) siRNA. B: Quantification of electron micrographs in HeLa-ATCC cells transfected with ns or dyn2 siRNA infected with Ad35 for 60 min. In dyn2 siRNA transfected cells significantly more Ad35 remained at the plasma membrane. Adsmooth, virus at the plasma membrane, AdEndtot, total endosomal virus, AdCyt, cytosolic virus.

Further, siRNA against clathrin heavy chain and the clathrin lymphoid myeloid leukaemia (CALM) protein were used to study the involvement of clathrin-mediated endocytosis in Ad35-infection in more detail. Clathrin heavy chain builds the clathrin triskelion together with the light chain and the clathrin assembly lymphoid myeloid leukemia (CALM) protein is a clathrin associated protein that plays an is important role in regulation of clathrin internalization machinery (Dreyling et al., 1996; Tebar et al., 1999).

Interestingly, both siRNAs reduced the infection of Ad35-eGFP in HeLa-ATCC, showing that dynamin and clathrin endocytosis is involved in Ad35 infection in these cells (fig. 5B, fig. 6B, respectively). Electron micrographs of cells treated with either dynamin or CALM siRNA 60 min p.i. revealed that Ad35 uses partially dynamin- and clathrin-mediated endocytosis for internalization (fig. 4B and fig. 6C). Further, investigation of nuclear targeting of Ad35-TR and Ad2-TR in HeLa-ATCC cells treated with siRNA against clathrin heavy chain or CALM showed that both viruses need functional clathrin mediated endoctosis for proper nuclear targeting (fig. 5D and fig. 6D). Taken together we conclude that clathrin-mediated endocytosis plays a role in the infection of Ad35 in certain cell types, either directly in uptake or indirectly like e.g. in the infection of Ad3. The indirect clathrin dependence could be

caused by destabilization of macropinosomes which are likely supported by clathrin-mediated endocytosis (e.g. fluid phase uptake stimulated by either Ad3 or Ad2 is decreased upon clathrin depletion of HeLa ATCC cells (first part of thesis and data not shown)). Clathrin might play a role in stabilization of the macropinosomes or support macropinoctosis by delivering lipids to other membrane components. Further, trafficking within the cells might be affected by clathrin depletion which might affect macropinosome maturation.

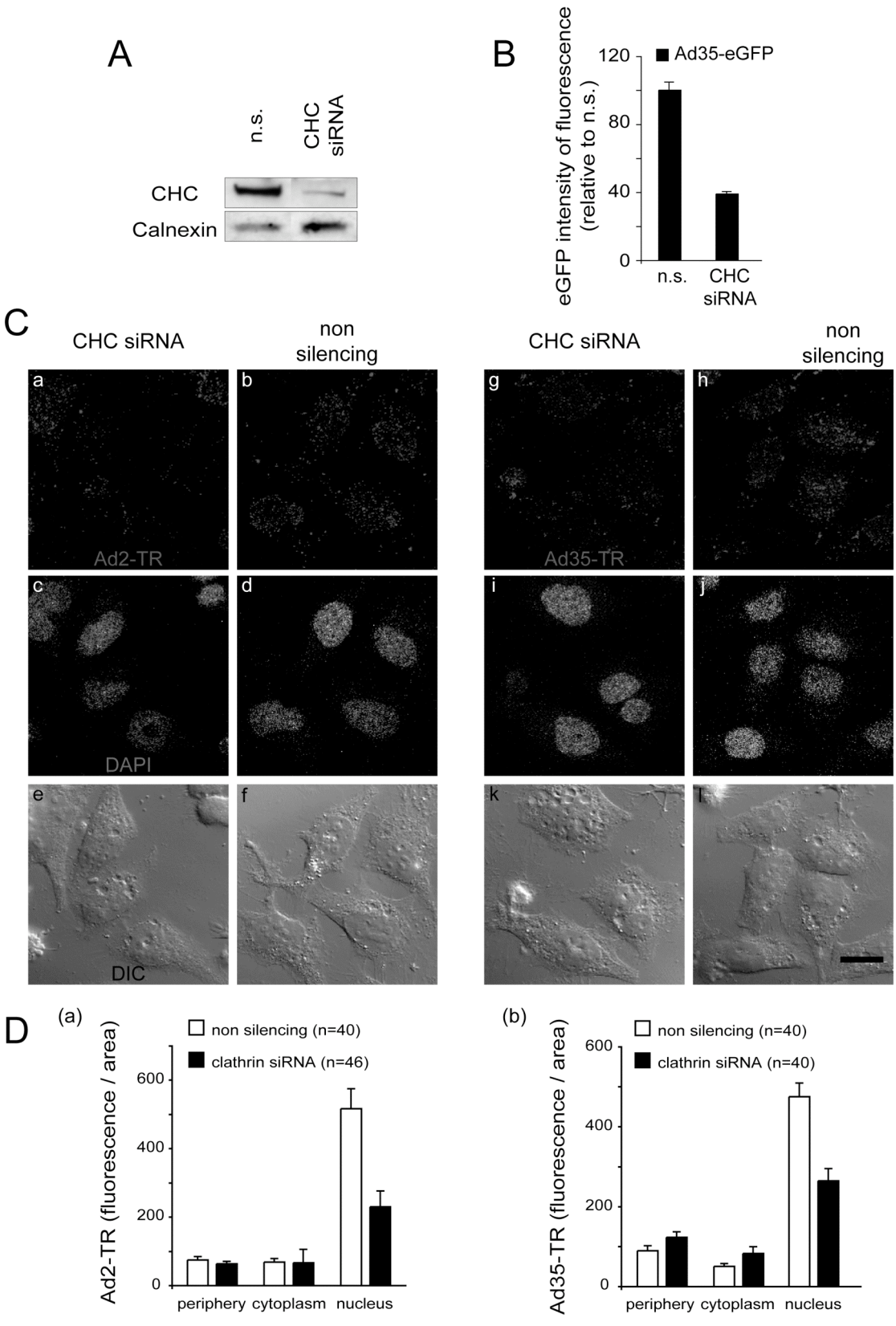


Figure 5. Clathrin dependent infection and nuclear targeting of Ad35 and Ad2 in HeLa-ATCC cells. A: Western blot of HeLa-ATCC cell lysates transfected with non-silencing (n.s.) or clathrin heavy chain (CHC) siRNA probed against clathrin heavy chain and calnexin for loading control. Knockdown was >90% compared to non-silencing cells. B: Ad35-eGFP transduction (6h) in n.s. or CHC siRNA treated cells. C, D: Nuclear targeting of Ad2-TR and Ad35-TR in n.s. or CHC siRNA treated cells. C: a,b,g,h TR-virus, respectively; c,d,I,j DAPI; e,f,k,l DIC. Bars = 20 µM. D: Quantification of nuclear targeting by Matlab analysis.

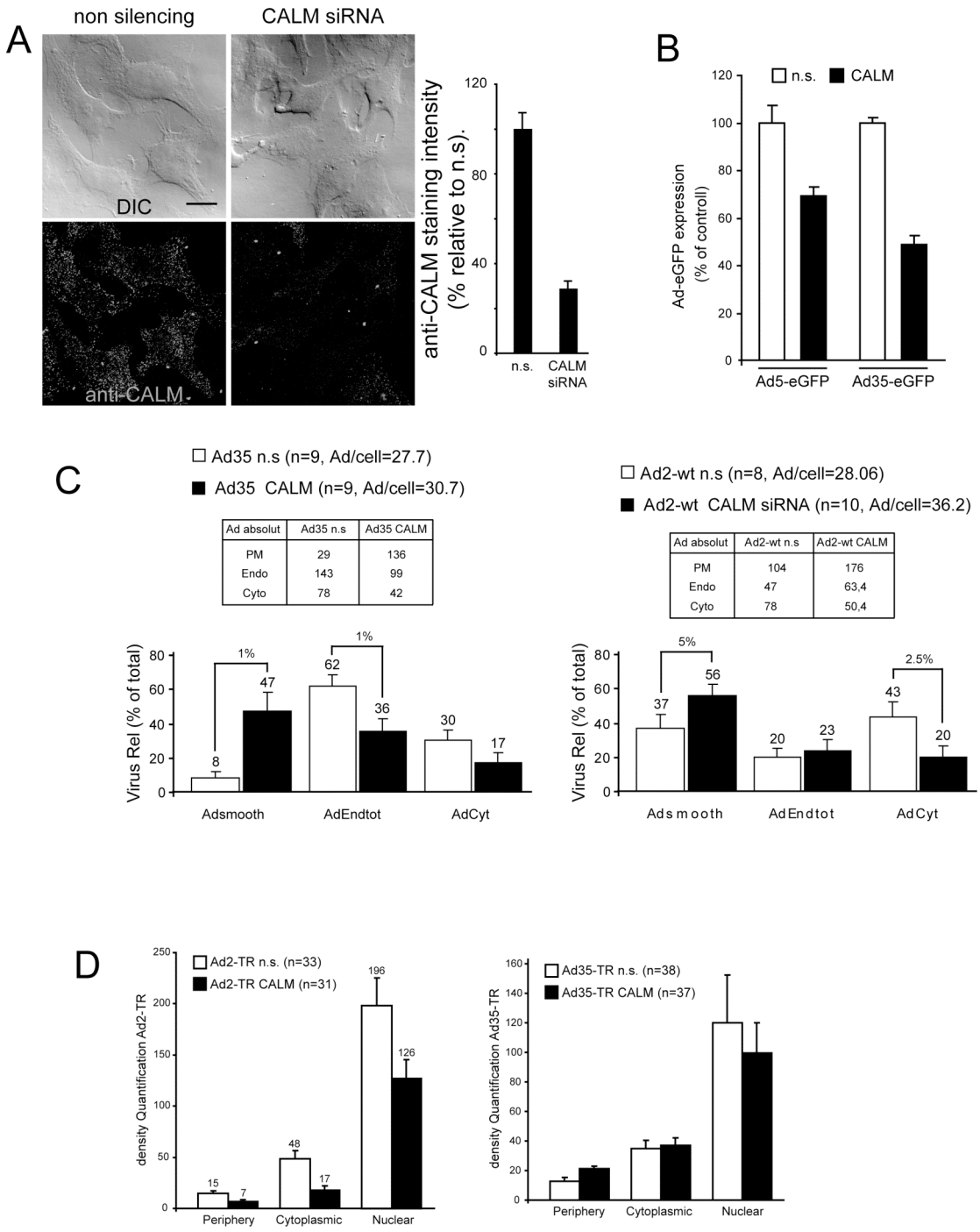


Figure 6. CALM dependent infection and nuclear targeting of Ad35 and Ad2 in HeLa-ATCC cells. A: Quantification of CALM immunofluorescence in HeLa-ATCC cells treated with non-silencing (ns) and CALM (CALM) siRNA. Bars=20µM B: Ad5-eGFP and Ad35-eGFP transduction in HeLa-ATCC cells treated with ns or CALM siRNA, respectively. C: Quantitative electron micrographs of both Ad35 and Ad2 infected cells for 60 min p.i. Adsmooth, virus at the plasma membrane, AdEndtot, total endosomal virus, AdCyt, cytosolic virus. Nuclear targeting of Ad2-TR and Ad35-TR in CALM or ns treated cells, respectively. D: Nuclear targeting of Ad2-TR and Ad35-TR in n.s. or CALM siRNA treated cells.

It was shown that Ad3 requires CtBP1 for infection (first part of this thesis). Since Ad35 enters HeLa-ATCC cells similar to Ad3 in fluid phase rich endosomes and clathrin coated pits, the requirement for the macropinosomal regulators CtBP1 and PAK1 in Ad35 infection in HeLa-ATCC cells was tested. HeLa-ATCC cells depleted of CtBP1 with two different siRNA showed a decrease of Ad35-eGFP infection measured by FACS analysis 6h p.i. To measure the knockdown level a quantitative immunofluorescence was performed on the Ad35-eGFP infected cells using a commercial available CtBP1 antibody. In comparison to non-silencing siRNA treated cells the CtBP1 signal was reduced to 18 % (R1 condition) or 9 % (R2 siRNA), respectively (figure 7). Infection with Ad35-eGFP was strongly reduced whereas Ad5-eGFP infection remained unaffected in these cells. Similar results were obtained by FACS-measurement analyzing more then 10000 cells per condition in triplicates (figure 8A). The requirement for PAK1 was studied in HeLa-ATCC cells and similar to Ad3 (first part of thesis) Ad35 infection is dependent on PAK1 (Figure 8) in both epithelial HeLa-ATCC and A549 cells.

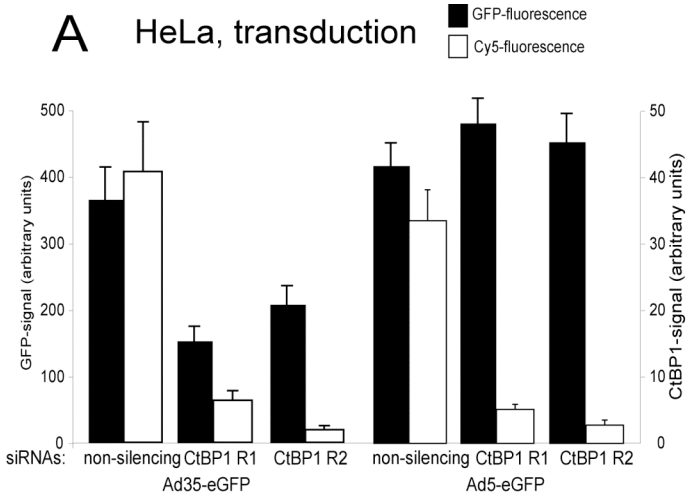


Figure 7. siRNA knock down of CtBP1 reduced infection of Ad35 but not Ad5. A: HeLa-ATCC cells transfected with CtBP1 siRNA R1 or R2 were infected with Ad35-eGFP or Ad5-eGFP, stained with an anti-CtBP1 antibody and analyzed for GFP expression and Cy5 fluorescence of CtBP1 upon indirect immunostaining by confocal analysis.

Like Ad3 (first part of thesis), Ad35 infectious entry is strongly dependent on CtBP1 and to some extent on dynamin and clathrin in HeLa-ATCC cells. A similar result was obtained by the phagocytic uptake of listeria bacteria requiring clathrin and the infectious uptake of Ad3 in HeLa-ATCC (Veiga,

2006, first part of thesis). Slight differences were obtained with Ad35 infection in A549 cells. Here, Ad35-transduction was dependent on PAK1, CtBP1 and to a lesser extent on dynamin or clathrin (figure 8B). Since hematopoetic cells are a favourite target for species B derived vectors we tested the CtBP1 and or clathrin dependece in the hematopoietic cell line K562. Ad35 infection was dependent on CtBP1 but not clathrin (figure 4C), whereas Ad5 infection was dependent on clathrin but not on CtBP1 (data shown in the first part of the thesis). Interestingly, in this cell line integrins are involved in the infection of Ad35, not in primary binding but as secondary receptors (Murakami et al., 2007).

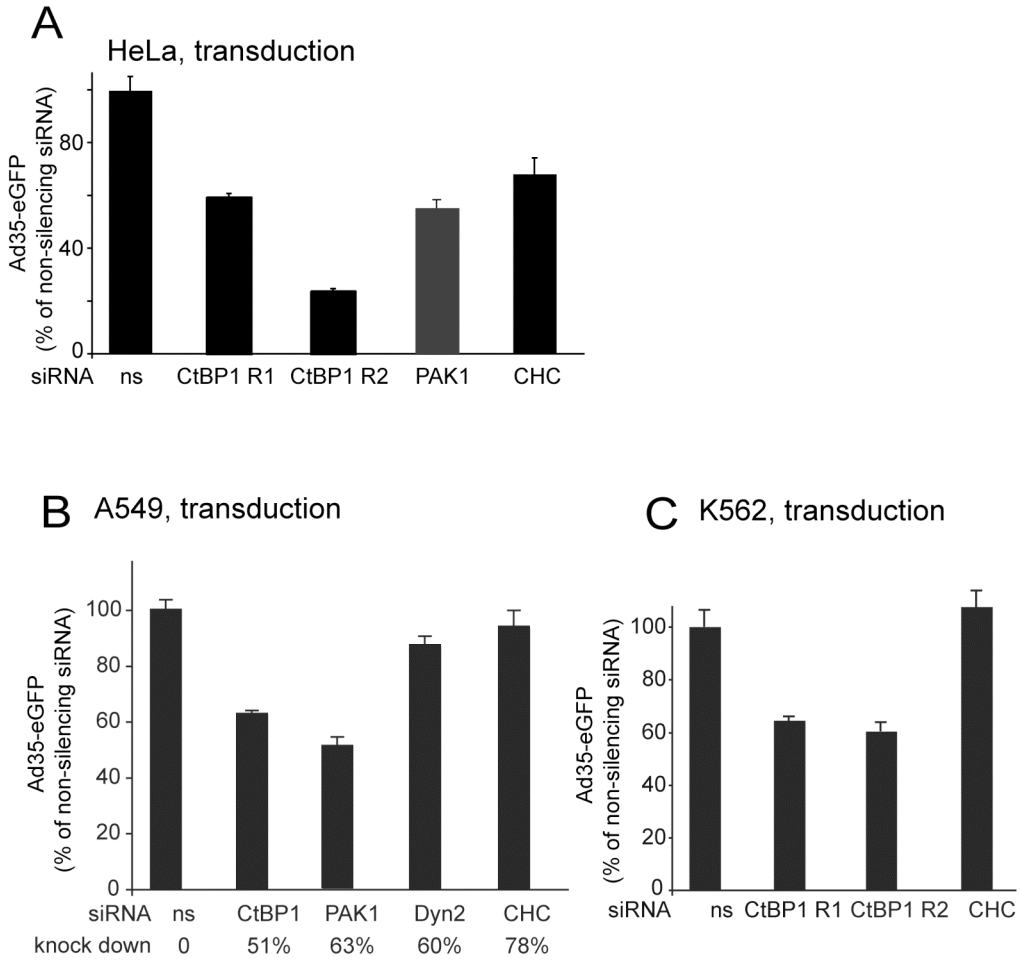


Figure 8. Different requirement of clathrin and dynamin, but not CtBP1 and PAK1 in different epithelial and hematopoietic cell lines in Ad35 infection. A: HeLa-ATCC cells transfected with CtBP1 siRNA R1 or R2, PAK1 siRNA or clathrin heavy chain (CHC) siRNA were infected with Ad35-eGFP and analyzed for GFP expression by flow cytometry. B: A549 cells were transfected with siRNA against CtBP1, PAK1, dynamin2 and clathrin heavy chain, infected with Ad35-eGFP and analyzed for GFP expression by flow cytometry. C: K562 cells were transfected with siRNA R1 or R2 against CtBP1 and clathrin heavy chain, infected with Ad35-eGFP and analyzed for GFP expression by flow cytometry.

Discussion

The data in the second part of this PhD thesis reveal that induced macropinocytosis, and to some extent, clathrin-mediated endocytosis is an infectious entry route for species B human adenovirus Ad35 in both epithelial and hematopoietic cells. Induction of macropinocytosis is a common mechanism of both species B Adenovirus Ad3, Ad7 and Ad35 and species C Ad2 and Ad5 (Meier et al., 2002, figure 1B and first part of this thesis), and, since macropinosomes release their content, show similar intracellular processing (data not shown). It is triggered most likely through binding of alphav integrins to RGD-motif in the penton base (first and second part of thesis, Nemerow 1993 and Meier and Greber, 2003), or possibly clustering of the primary receptor, CD46. Like viruses of the species C that enter epithelial cells by dynamin-dependent endocytosis (Meier et al., 2002; Gastaldelli and Greber, unpublished data) and do not require the macropinocytosis regulators PAK1 and CtBP1 (first part of thesis), Ad35 infection is dynamin dependent to some extent and needs PAK1 and CtBP1, similar to species B Ad3 infection. Most likely, the entry of Ad35 is similar to the one of Ad3, since both viruses endocytose with fluid phase marker BSA-gold in deep invaginations containing a high number of viral particles. These invaginations are numerous at around 7 min post infection, when fluid phase uptake is maximal (data not shown). Species C Ad2 or Ad2 ts1 do not show the typical invaginations filled with virus particles in the first 15 min of infection. Noteably, a small amount of Ad35 is found in coated pits, possibly pointing towards an additional uptake pathway for this virus.

Ad35 binds CD46 as a primary attachment receptor. This molecule has been referred to as a pathogens magnet since many different pathogens use it as primary attachment to cells, including measles virus (Edmonton strain), human herpesvirus 6, Steptococcus pyogenes and pathogenic Neisseria (Cattaneo, 2004). CD46 is probably attractive for pathogens because it links innate and acquired immune function, and impairs activation and effector function of T-cells, and T-cell response to NK cells (Oliaro et al., 2006). In addition, CD46 is expressed on all nucleated human cells, and plays an

important regulatory role in complement mediated cell lysis. The complement cascade is a phylogenetically ancient part of innate immunity, and controls bacterial and viral infections (Carroll, 1998). CD46 signalling is also intimately linked to endocytosis. Depending on the degree of ligand crosslinking, it engages in membrane ruffling, macropinocytosis or constitutive clathrin-mediated endocytosis (Crimeen-Irwin et al., 2003). Possibly extensive crosslinking of CD46 through the multivalency of the Ad35 fibers leads to pseudopode formation and membrane ruffles, and gives rise to Ad35 bearing vesicles that are delivered towards degradation in late endosomes and lysosomes. It might be that viruses found in coated pits were not able to crosslink CD46 molecules extensively or, also likely, bound to integrins and were taken up in a single-virus carrying vesicle similar to e.g. the uptake of species C Ad2 and Ad5 (Meier et al., 2002).

Endosomal escape of Ad35 is considerably slower than Ad2 (t½ of 20-30 min compared to 15 min of Ad2, Greber et al., 1993), although the uptake rates at high moi in transmission electron micrograph quantifications seemed to be equal (data not shown). This difference may relate to the differential receptor usage of Ad2 and Ad35, e.g. CAR versus CD46, different secondary integrin coreceptors, or virus specific factors.

Macropinocytosis has strict requirements of actin, RhoGTPases Rac1 and Cdc42, protein kinase C, and the sodium/proton exchanger extruding protons from the cytosol. Proton efflux from the cell is an evolutionarily conserved mechanism to regulate cytoskeleton dynamics, cell migration, and growth factor induced proliferation. Ad35 macropinocytosis requires PAK1 similar to Ad3, a key factor in the actin remodeling network (Parrini et al., 2005). Activated PAK1 phosphorylates many downstream targets. Such a target is CtBP1, a transcriptional coreceptor involved in membrane transport and dynamin-independent endocytosis as well as exocytosis (Bonazzi et al., 2005). PAK1 disrupts CtBP1 dimers, and triggers CtBP1 translocation to the cytoplasm which derepresses transcription (Barnes et al., 2003). The

observations that Ad35, Ad3 and Ad2/5 activate PAK1, and that CtBP1 is found on Ad35/3 or Ad2/5 induced macropinosomes might suggest that the recruitment of CtBP1 to macropinosomal membranes contributes to derepression of host genes during viral entry. It might be a conserved mechanism of adenovirus to prepare the host cell already at the cell surface.

Ad35 infects basically the urinary tract and the kidney. Since we found no significant difference in uptake of Ad35 in comparison to Ad3 we conclude that the different tropism in respect to Ad3 that infects primarily respiratory organs is caused by a later step in infection. It might be that Ad35 can infect respiratory epithelial cells since immunocompromised individuals show Ad35 infections, but probably in healthy individuals it might efficiently be removed from the immunsystem, whereas Ad3 can resist better to this defence mechanisms.

However, more detailed investigation about the entry of Ad35 in both epithelial and hematopoietic cells are needed. Interesting would be, e.g. to test the clustering of CD46 and/or integrins by binding of specific primary antibodies and undergo clustering by secondary antibody binding. Such a system could be used to study whether CD46/integrin clustering is sufficient to undergo fluid phase uptake stimulation similar to the process that is induced by the virus. Further studies are needed to investigate, whether CtBP1 phosphorylation by PAK1 is needed for CtBP1 activation and whether the transcription is changed upon viral macropinocytosis induction (e.g. it is known that adenovirus change transcription of the host, but it is not known whether this occurs during entry of the virus; (Endter and Dobner, 2004; Wold et al., 1994; Higashimoto et al., 2006)).

Material and Methods

Cells and viruses

Cells were grown in DME (GIBCO-BRL) containing 10% FCS (GIBCO-BRL) at low passage number as described (Meier et al., 2002). Human melanoma M21 litter (negative for surface-expressed αv integrins) and M21L cells (positive for cell surface αv integrins) were from Dr. D. Cheresh (Scripps Research Institute, La Jolla, CA). K562 chronic myelogenous leukemia cells were grown as described (Meier et al., 2005). Ad35, Ad7 and Ad5 were grown, labeled and isolated as described (Meier et al., 2002; Suomalainen et al., 2001). We estimated that 1000 Ad35 particles were equivalent to 2 infectious particles bound per cell (moi 2), based on a particle to infectious particle ratio of about 20 (typical of adenoviruses), and the observation that 4% of radiolabeled Ad35 bound to cultured human epithelial cells in any given experiment.

cDNAs

K44A-dyn2 and dyn2 wt expression plasmid were from Dr. C. Lamaze (Pasteur Institute, Paris, France).

Fluid phase uptake and Ad35-eGFP transductions

Dextran uptake was carried out as described (Meier et al., 2002). eGFP transductions and analyses by flow cytometry or single cell confocal microscopy were carried out as described (Meier et al., 2005).

Fluorescence microscopy, immunofluorescence and nuclear targeting

Cells were transfected with different DNA constructs 40 h prior to experiment using Fugene 6 (Roche, according to manufacturer's instruction), infected with Ad35-eGFP or Ad5-eGFP at 37° C for 60 min, washed and incubated at 37° C for 15 h. Cells were fixed and mounted with DAKO. For dextran and transferrin uptake, cells were synchronized with 5 µg/ml of Ad35, Ad7 or Ad5 in the cold, washed warm and pulsed with a mixture of 0.5 mg/ml dextran-TR and 20 µg/ml of transferrin-Alexa647 in RPMI-BSA at 37° C for 30 min

(waterbath), followed by a 5 min chase, fixed and mounted with DAKO. Confocal laser scanning microscopy was performed on a Leica-DM SP2 RXA2-TCS-AOBS microscope (Leica Microsystems, Wetzlar, Germany) equipped with an Ar-ArKr laser, a He-Ne 543-594 laser, a He-Ne 633 laser, a diode laser at 405 nm, and a 63 x oil immersion objective (N.A. 1.4 PL APO). The pinhole value was 1.0, airy 1, yielding optical sections of ~0.48 µm with a voxel of 0.233 by 0.233 by 0.48 µm. The zoom factor was 2. Image processing was performed with Leica and Photoshop software (Adobe), and fluorescence intensities determined using Image J (http://rsb.info.nih.gov/ij/) on cell total projections. For nuclear targeting Ad2-TR was internalized for 90 min, Ad35-TR for 120 min, respectively. Cells were fixed, nuclei stained with DAPI, and CLSM sections with a z-difference of 0.5 µm were taken. Quantification of cellular distribution of Ad-TR was performed using a Matlab-based program made by P. Schönenberger.

siRNA transfections

K562 cells were transfected with siRNA directed against clathrin heavy chain (AACCUGCGGUCUGGAGUCAAC; Qiagen, (Meier et al., 2005)) and against CtBP1 (CCGUCAAGCAGAUGAGACAUU; GGAUAGAGACCACGCCAGUUU (Dharmacon) (Bonazzi et al., 2005)) using Nucleofector I (Amaxa; program T-03) according to the manufacturer's instructions, using non-silencing siRNA sequences (Qiagen, or Dharmacon) as controls. Transfections were done at day 0 and day 2, cell lysates for Western blotting and experiments were collected at day 4. HeLa cells were transfected with siRNA directed against dynamin2 (GACAUGAUCCUGCAGUUCA, Qiagen), clathrin heavy chain, CtBP1 or PAK1 (validated siRNA Cat. SI00605703 and SI00605696; Qiagen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfections were done twice at day 0 and day 2, cell lysates for Western blotting and experiments collected at day 4. A549 cells were transfected with siRNA directed against clathrin heavy chain, CtBP1, PAK1 or dynamin2 using Lipofectamine 2000.

Preparation of BSA-gold and Transmission electron microscopy

15 nm collodial gold was prepared by citrate reduction of $HAuCl_4$ (Horisberger and Rosset, 1977). To 20 ml of collodial gold solution (pH adjusted to 5.9) 50 µl of 10 mg/ml BSA (Sigma, fatty acid free) solution was added (De Roe et al., 1987). To stabilize the BSA-gold complex, 1ml of 1% PEG 20000 (Roth, Switzerland) were added, the sample centrifuged at 28'000 g for 60 min, and the pellet dissolved in 2 ml gold-buffer (sterile filtered PBS containing 0.2% PEG-20000) and stored at 4° C. BSA-gold internalization was performed after cold binding of Ad3 or Ad2-ts1 using a 1:1 dilution of BSA-gold with RPMI-BSA (approximately 0.1 mg/ml of BSA) at 37° C for 10 min. After cold binding of Ad35 or Ad2 (50 µg/ml, moi 5000) for 60 min, washing and internalization as appropriate, cells were fixed in 2% formaldehyde-1.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 (CaCo) overnight, and washed several times in CaCo, followed by postfixation in 1% OsO_4 (Electron Microscopa Sciences) and 1.5% potassium ferricyanide ($FeK_3(CN)_6$) in double distilled water at 4° C for 60 min (modified according to the medhod of Simionescu and Siminonescu). Specimens were rinsed in 0.1 M sodium cacodylate, contrasted with 1% tannic acid in 0.05 M sodium cacodylate at room temperature for 45 min, washed in 1% sodium sulfate, rinsed in H_2O, stained in 2% uranylacetate in H_2O over night, and embedded in Epon as described previously (Meier et al., 2005). Virus particles were quantified at 50000 x magnification in ultrathin sections at the plasma membrane, endosomes and cytosol, and viewed in a transmission electron microscope (Zeiss EM 902A) at an acceleration voltage of 80,000 V.

Acknowledgements

We thank Menzo Havenga (Crucell, The Netherlands) for Ad35-eGFP. T. Honegger for preparing BSA-gold. P. Schönenberger for the Matlab based nuclear targeting analysis program.

Final conclusion and remarks

In this thesis, the endocytic uptake mechanisms involved in species B adenovirus Ad3 and Ad35 entry were studied. A novel pathway taken for viral infection was discovered involving surface receptors CD46 and integrins, signalling through Rac1, PAK1 and the transcriptional regulator CtBP1. Internalization of the species B viruses is associated with fluid phase uptake, and endosome rupture releases both fluid phase and virus to the cytosol. The viral induction of macropinocytosis is hijacked by Ad3 and Ad35 to gain access to both epithelial and hematopoietic cells. Interestingly, CtBP1, a transcriptional regulator, is present on macropinosomes and we conclude that these endosomes, rich in fluid phase and viral particles, are signalling platforms, inducing transcriptional changes to prepare viral replication already at the beginning of infection during entry. They are stimulated by adenoviruses of both species B and C and might be used to manipulate and change transcription, e.g. host anti-viral defense might be downregulated, cell polarity could be changed by lower expression of e.g. E-cadherin, and many other different changes might be stimulated by Adenovirus during entry.

After both species B 1 Ad3 and species B2 Ad35 bind CD46 integrins are recruited. Integrins are thought to be coreceptors which might play a role in uptake and signaling (Meier and Greber, 2003). Given that differences in binding between Ad3 and Ad35 to the melanoma cells were found (first and second part of this thesis) and our surface trypsinization experiments in HeLa cells revealed a different behaviour between Ad3 and Ad35, this might suggest that binding to CD46 differs between Ad3 and Ad35. In addition, integrin coreceptors or cofactors of unknown nature might be recruited by different Ads. In support of this notion, two uptake mechanisms taken by Ad35 are clearly distinct – the first one a dynamin and clathrin dependent mechanism and the other one PAK1 and CtBP1 dependent macropinocytosis. Macropinocytosis is used by both Ad3 and Ad35. These viruses are found in characteristic tubular vesicles within the first few minutes of entry. The tubular endosomal structures disappear within the first 30 minutes of infection,

maturating into endosomes with a large number of viral particles, or the viruses escape out of these endosomes to the cytosol. It is an important question to investigate where the membrane is recruited from and what the exact compostition of these endosomes is. This would most likely require the isolation of macropinosomes.

The different tropism of Ad3 or Ad35 might be explained by partly different entry requirements downstream of CD46 and integrins. Interestingly, immunocompromised patients, e.g. AIDS- or cancer patients, show Ad35 infection whereas healthy individuals do not indicative of portent immune control of Ad35 infections (Hierholzer 1992).

Since different primary attachment receptors, but similar secondary receptors are used in both species B and C Ads endocytosis we conclude that the known secondary receptors alone might not determine the type of endocytic pathway that is taken by a certain Ad. More likely, the primary receptor binding is the factor needed for the initial binding, and determines together with the secondary receptor the uptake mechanism. This is supported by the study of Miyazawa et al., 1999 showing that a so-called fiber-swapped virus, a Ad5 derived capsid bearing a fiber protein replaced by a fiber of Ad7, was targeted to an intracellular route different from the wildtype Ad5 route but similar to Ad7 pathway. The uptake efficiency was similar but the intracellular pattern of these viruses showed a rapid translocation of Ad5 to the nucleus, whereas both Ad7 and the fiber-swapped virus Ad5/F7 were less efficiently transported to the nucleus and partially ended up in late endosomes/lysosomes. However, the nature of the fluid phase uptake stimulation is very likely to be caused by integrins since both species B and C Ads stimulate macropinocytosis with similar kinetics, and trigger macropinosomal content release into the cytosol with similar kinetics.

OUTLOOK

Additional investigations are needed on the endocytic uptake. One of the most interesting questions to clarify is the mechanism of the endosomal escape of adenoviruses. Given that no membrane surrounds these viral particles, a simple fusion mechanism between viral proteins and endosomal membrane, like e.g. influenzavirus glycoprotein undergoing low endosomal pH induced strucutral changes and subsequent fusion with the limiting membrane, is unlikely to occur in adenovirus entry. More likely, signalling events caused by activated integrins might be the important lysis trigger of endosomal membranes and subsequent viral release. However, a role of viral proteins in the endosomal escape is possible, e.g. the protein VI containing a lymphopatic helix that could be inserted into the endosomal membrane (Wiethoff et al., 2005). Further experiments should investigate with the role of protein VI and others in membrane lysis.

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Abbreviations

Ad	human adenovirus
AP2	adaptor protein 2
Arp2/3	actin related protein 2/3
CALM	Clathrin assembly lymphoid leukemia
CAR	coxsackie B virus and adenovirus receptor
CCP	complement control protein repeat
CCV	clathrin coated vesicle
Cdc42	cell division cylce 42 protein
CHC	clathrin heavy chain
CLSM	confocal laser scanning microscopy
CNX	calnexin
CME	clathrin-mediated endocytosis
CtBP1-L	C-terminal binding protein long
CtBP1-S	C-terminal binding protein short
CTX	cholera toxin B
DAG	diacylglycerole
DAPI	4'-6'-diamidino-2-phenylindole
DIC	differential interference contrast
EE	early endosome
EEA1	early endosome antigen 1
EGF	epidermal growth factor protein
EIPA	N,N-ethyl-isopropoyl-amiloride
EM	electron microscopy
Eps15	epidermal growth factor receptor pathway substrate 15
ESCRT	endosomal sorting complex required for transport
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
GEEC	GPI-anchored protein enriched early endsosomal compartment
GPI	Glycophosphatidylinositol
HIV	human immunodeficiency virus
HRS	hepatocyte growth factor-regulated tyrosine kinase
LBPA	lysobiphosphatidic acid
LDL	low density lipoprotein
LE	late endosome
LY	lysosome
MOI	multiplicity of infection
MTOC	microtubule-organizing center
MVB	multi-vesicular bodies
NSF	N-ethyl maleimide-sensitive factor
PAK1	p21-activated kinase
PDGF	platelet-derived growth factor
p.i.	post infection
PH domain	pleckstrin homology domain
PI3K	phosphatidylinositide-3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate
Rac1	ras-related C3 botulinum toxin substrate 1
RE	recycling endosome
RGD	amino acid abbreviation for arginine-glycine-aspartate
SNARE	SNAP receptors
SNAP	soluble NSF attachment protein
SV40	simian virus 40
Tfn	transferrin

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1997 – 2000	Undergraduate student at the ETH Zürich, Biochemistry and Molecular Biology, Microbiology, Bioorganic Chemistry and Biotechnology
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2004	Course in Advanced Microscopy, University of Zurich.
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2000	4 months fulltime practical work at the “Firma Geistlich Agrasana”, Schlieren. Project “Removal of H_2S out of waste gas”.
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INVITED TALKS

- The 8th International Adenovirus Meeting. August 30th - September 2nd, 2006, Zurich, Switzerland. “Macropinocytic uptake and infection of epithelial and hematopoietic cells by human Adenovirus serotype 3”.

- Zoology Institute talk May 5th 2006. “How Adenoviruses trick cells”

POSTER

- ELSO. European Life Scientist Organization. Dresden, Germany. September 3rd - 6th 2005. “Macropinocytic uptake and infection of human Adenovirus type 3 in epithelial and hematopoietic cells”.

SCHOLARSHIPS

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